REMARKS

I. Preliminary remarks

Claims 25, 28 and 31 are amended herein. The amendments to claims 25, 28 and 31 correct typographical errors that would have been apparent to one of skill in the art, or otherwise clarify the subject matter claimed in a manner consistent with the original disclosure in the application as filed. Accordingly, no new matter has been introduced by these amendments.

In the remarks below, several references are cited and copies of those references are provided in Exhibit A, attached hereto. In particular, copies of Blachere et al., J. Exp. Med. 186:1315 (1997), Ishii et al., J. Immunol. 162:1303 (1999), Belli et al., J. Clin. Oncol. 20:4169 (2002), Calderwood et al., Eur. J. Immunol. 35:2518 (2005), and U.S. Patent Application Publication Nos. 20020187159, 20030129196, and 20040047876 are attached as Exhibit A.

II. The rejection of claims 21 and 22-31 under 35 U.S.C. § 112, second paragraph, has been overcome

A. "A non-mammalian heat shock response"

The Examiner rejected claims 21 and 22-31 under § 112, second paragraph, for asserted indefiniteness in reciting "non-mammalian heat shock response" and in assertedly failing to define the metes and bounds of the stimulus causing it. As a preliminary matter, Applicant notes that claim 22 was canceled in amendment A filed June 15, 2005. Accordingly, the rejection is being treated as a rejection of claims 21 and 23-31. In response, Applicant traverses.

Applicant submits that the term "non-mammalian heat shock response" would be clear to the person of ordinary skill in the art. At page 5, lines 17-20 for example, the application recites that "the present invention also provides a composition comprising a heat shock protein (hsp) derived from a non-mammalian eukaryote coupled to a heterologous antigenic polypeptide which composition is capable of inducing an immune response to said antigenic polypeptide in a mammal." A method for obtaining the composition is disclosed at page 6, lines 5-9 of the application. The two-step method involves (1) expressing the

antigenic polypeptide in a non-mammalian cell subjected to a stimulus which causes the induction of a heat shock response in that cell, and (2) recovering the antigenic polypeptide coupled to one or more hsps from the cell. The method is further explained in the application at lines 10-20 of page 6. A nucleic acid encoding the antigenic polypeptide is introduced into the non-mammalian cell; expression of the polypeptide is realized by culturing the cell under conditions providing for that expression, the stimulus is then defined by noting that the cell is exposed to "a stress that is capable of initiating the production of heat shock proteins in that cell", and the expressed complex is recovered. At page 8, lines 15-20, the application discloses that heat shock proteins are found in both prokaryotic and eukaryotic cells. In particular, the application states that "it is preferred that the hsps are derived from non-mammalian cells." Thus, a "non-mammalian heat shock response" is unambiguously defined in the application as the response of a non-mammalian cell to a stress, such as raised temperature, with that response involving the expression of at least one heat shock protein.

The skilled person would immediately understand that such a term relates to the response of a cell to the treatment of that cell with a stress such as a raised temperature, i.e., to the production by the cell of hsp when the cell is exposed to raised temperatures. The exact conditions (e.g., temperature, time) which result in a non-mammalian heat shock response in a cell may vary depending on the cell type involved. However, the temperature at which a response is generated is easily and routinely determined by the skilled person, simply by heating the cell and looking at the increase in hsp production against rising temperature. For example, at page 14 lines 19-22, the method of determining the optimal expression of hsp by exposing cells to differing temperatures for different periods of time is described. Furthermore, in Example 2 at page 46 line 11-15, the specification describes exposing Sf9 cells to differing periods at 43°C to establish the optimal exposure time for hsp expression.

For the foregoing reasons, Applicant submits that the term "non-mammalian heat shock response" is not indefinite under 35 U.S.C. § 112, second paragraph. Accordingly, this basis for rejecting the claims has been overcome.

B. "Derivative" and "fragment"

The Examiner rejected claims 25-28 under 35 U.S.C. § 112, second paragraph, for effectively reciting the assertedly indefinite terms "derivative" and "fragment." In particular, claim 25 recites that the antigenic polypeptide may be "an antigen of a pathogenic organism, or a fragment or derivative thereof." Claims 26-28 ultimately depend from claim 25, and thereby incorporate this feature in the subject matter claimed. The Examiner has acknowledged that "antigenic polypeptide" is not indefinite in stating that "[t]he metes and bounds of antigenic peptide or polypeptide are clear." (Office Action at page 6.) Thus, the indefiniteness arises in defining the meaning of an antigen, or a fragment or derivative thereof. Although Applicant maintains the position that an "antigen" of a pathogenic organism, and a "fragment" or "derivative" of such an antigen, have clear and unambiguous meanings, the issue is moot in view of the Examiner's and Applicant's agreement that "antigenic polypeptide" is not indefinite. The claimed invention is drawn to methods of producing an immunogenic complex comprising a heat shock protein (hsp) coupled to a heterologous antigenic polypeptide. Whether a "fragment" of an antigen is itself an antigen, and therefore whther a fragment of an antigen fragment is a definite term, does not disturb the agreed definiteness of the claimed invention, drawn to methods of producing complexes of hsps and antigenic polypeptides. Similarly, the asserted indefiniteness of an antigen "derivative" in failing to define the degree of change embraced by an antigen derivative, is also not relevant to the claimed methods. Accordingly, Applicant has canceled claim 25 and revised the dependencies of claims 26-28 without altering the scope of the latter claims and without surrendering any subject matter in response to an issue of patentability. Applicant has eliminated a distracting issue in the interest of expediting the prosecution of claims that have always been drawn to methods of producing antigenic complexes of hsps and antigenic polypeptides.

For the foregoing reasons, Applicant submits that the rejection of claims 25-28 under 35 U.S.C. § 112, second paragraph, for indefiniteness has been rendered moot and should be withdrawn.

III. The rejection of claims 21-31 under 35 U.S.C. § 112, first paragraph, for lack of written descriptive support, has been overcome

The Examiner rejected claims 21 and 22-31 (i.e., 21-31) under § 112, first paragraph, for asserted lack of written descriptive support. In supporting remarks, the Examiner requests citations to the specification that provide support for a "non-mammalian heat shock response." In response, Applicant traverses.

As an initial matter, Applicant submits that the Examiner intended to reject claims 21 and 23-31 because claim 22 had been canceled and Applicant addresses the instant rejection on this basis. The standard for written descriptive support under § 112, first paragraph, does not require ipsis verbis support. In the Summary of the Invention section at page 4, lines 4-11, the application recites a production method according to the invention, reciting in pertinent part that "the antigenic polypeptide [is expressed] in a cell which cell has been subjected to a stimulus which causes the induction of a heat shock response in said cells." In the very next sentence spanning lines 12-13, the application recites that "[p]referably the cell is a non-mammalian eukaryotic cell, more preferably an insect cell." Further, at page 8, lines 15-20, the application recites that "the hsps present in the hsp/antigen complex may be from any source, prokaryotic or eukaryotic. However, it is preferred that the **hsps are derived from non-mammalian cells**, more preferably non-mammalian eukaryotic cells such as insect cells." (Emphasis added in bold.)

The above-quoted passages from the original application provide the requested passages showing that the inventors had possession of the claimed subject matter as of the effective filing date. Thus, the application-as-filed provides written descriptive support for the subject matter being claimed in accordance with § 112, first paragraph. Accordingly, the rejection of claims 21 and 23-31 under § 112, first paragraph, for asserted lack of written descriptive support has been overcome and should be withdrawn.

IV. The rejection of claims 21, 23-24, and 29-31 under 35 U.S.C. § 103(a) over Srivastava has been overcome

The Examiner rejected claims 21, 23-24, and 29-31 under § 103(a) over Srivastava, asserting that the reference taught a method of making an immunogenic complex comprising HSP and heterologous antigenic peptides in insect cells. In support, the Examiner asserted that one could express antigen-hsps complexes in insect cells using a baculovirus system with

an expectation of success in producing an antigen/non-mammalian hsps complex arising from the knowledge that "the elements are known to work." In response, Applicant traverses.

Srivastava indicates that insect cells may be used as potential host-vector cells for the expression of cancer antigens. However, the focus of Srivastava is on the expression and use of mammalian hsps in combination with cancer antigens. There is no clear and unmistakable teaching in Srivastava that the native hsps of the host insect cells may be used. In fact, in papers published concurrently and after the priority date of Srivastava, such as Blachere et al., J. Exp. Med. 186:1315 (1997) and Ishii et al., J. Immunol. 162:1303 (1999) (copies of which are enclosed), the only hsps which are used in association with cancer antigens to produce immunogenic complexes are mammalian hsps from mammalian cells.

It would not be obvious to the person of ordinary skill that insect hsps could be used to produce immunogenic complexes, as the property of the trafficking of complexed peptides was thought to be peculiar to mammalian hsps and a reflection of the role of the cellular hsps in the cell biology of antigen loading on MHC in Antigen Presenting Cells (APCs). The restriction to mammalian hsps is reflected in the focus of the cited Srivastava patent application, and also persists in more recent patent applications, such as US Patent Application Publication Nos. 20020187159, 20030129196, and 20040047876 (copies of which are enclosed). In these recent patent applications, the only hsps discussed are derived from mammalian cells, reflecting the then-current thinking that only mammalian hsps would be suitable for the generation of immunogenic complexes that may be used as vaccines.

A focus on the use of mammalian hsps to form complexes with antigenic peptides from cancer cells is also present in recent publications such as Belli et al., J. Clin. Oncol. 20:4169 (2002) and Calderwood et al., Eur. J. Immunol. 35:2518 (2005) (copies of which are also enclosed).

Thus, there is no teaching in Srivastava to direct the person of ordinary skill to view the mention of insect cells as potential vector-host cells as a direction to use the hsps from these cells as the hsps with which to form the hsps-antigenic peptide complex. The skilled person, given the teachings available at the time of filing of the application and the information presented in the Srivastava specification, would merely have viewed the mention of insect cells as being a potential vector-host cell for recombinant mammalian hsps expression (rather than heterologous insect hsps), as it was thought that mammalian hsps

were necessary for interaction of the hsps with APCs and, therefore, for the immunogenic effect.

In view of the disclosures of Srivastava characterized above, the Examiner's assertion that the antigen-hsps complexes of the invention "could be expressed" by baculovirus in insect cells is an unmistakable indication that the Examiner has engaged in the impermissible hindsight reconstruction of the invention using the application as a guide. (Office Action, pages 3-4; emphasis added.) Under the law, the issue is not whether a prior art disclosure could be modified to arrive at the invention, the issue is whether there is a proper motive or suggestion to modify a prior art disclosure to arrive at the invention, including each of its elements, with a reasonable expectation of success. The Examiner has not identified any motive or suggestion to modify the disclosure of Srivastava, and the description of Srivastava provided above establishes that such a motivation or suggestion will not be found because the art considered mammalian hsps to be essential to APC interaction and an immune response. In addition, the Examiner has not shown that it was known in the art that a non-mammalian hsps would be useful in eliciting an immune response and, therefore, the Examiner has erred in finding a reasonable expectation of success because "the elements of the method are known to work." (Office Action, page 4.)

For all of the foregoing reasons, Applicant submits that the Examiner has not established that each limitation of any of the rejected claims is taught or suggested in the cited reference, has not identified any motive or suggestion to modify the teachings of the reference, and has not established a reasonable expectation of success in arriving at the claimed subject matter. Accordingly, a *prima facie* case of obviousness for any of claims 21, 23-24, or 29-31 under 35 U.S.C. § 103(a) over Srivastava has not been established and the rejection should be withdrawn.

V. The rejection of claims 21 and 25-28 under 35 U.S.C. § 103(a) over Srivastava in view of Deregt has been overcome

The Examiner rejected claims 21 and 25-28 as obvious under § 103(a) over Srivastava in view of Deregt, asserting in support that Srivastava teaches the making of immunogenic complexes with HSPs and Deregt teaches that BVDV is a known bovine pathogen and the E2 region has been used in subunit vaccines. Motivation for combining the teachings was

assertedly found in Srivastava's disclosure that the complexes are immunogenic and because BVDV is a known, common virus of economic concern. In response, Applicant traverses.

The Examiner relied on the characterization of Srivastava provided in rejecting certain claims as obvious over that reference alone, and Applicant has addressed that characterization in section IV, above. As noted therein, the Examiner erred in failing to establish that each limitation of any of the pending claims had been disclosed or suggested in Srivastava because Srivastava is silent with respect to the belief in the art that mammalian hsps were required in order to interact with APCs and induce an immune response. Thus, Srivastava also fails to provide a motive or suggestion to modify the teachings therein to include antigen/non-mammlian hsps complexes, and fails to provide a reasonable expectation of success. The secondary reference, Deregt, does not remedy any of these defects, as established below.

Deregt et al. teaches that (a) E2 has neutralizing domains which can be recognized by antibodies, and that (b) a number of groups had previously studied the use of recombinant E2 antigens in vaccines to immunize cattle, sheep or pigs. Although Deregt pointed out that a number of studies had been carried out using sub-unit vaccines against pestiviruses, all of these papers in fact showed that the approach did not work in the studies carried out in ruminants.

Animals vaccinated with E2 antigens were then challenged with similar live pestiviruses to the derived E2 immunogenic proteins used in the vaccines. As stated in Deregt, "*[i]ts use as a component of subunit vaccines for BVDV in cattle and sheep, and CSFV in swine, have demonstrated that varying levels of protection against disease can be achieved by vaccination with E2.*" (Emphasis added.) In fact, in three of four studies quoted (all carried out in cattle and sheep), the levels of protection were very low, below the level of protection that would be acceptable for a commercial vaccine.

The only previously published study quoted in Deregt that showed any real level of recombinant E2-only protection was that carried out in pigs by van Rijn et al. (1996). Here, E2 derived from one CSFV isolate was used in a vaccine that gave protection only against lethal challenge with the same live CSFV isolate. Assessment of the effectiveness of the vaccine was based only on the development of homologous neutralizing antibodies and there was no measurement of fetal protection, as carried out in the present application.

Where fetal protection studies were carried out in sheep [for instance Bruschke et al. 1997)], there was incomplete protection even against homologous challenge. That is, the same live virus was used to challenge the vaccinated sheep as the recombinant E2 protein used in the vaccine, and the vaccination failed to protect the fetus.

Most importantly, in none of the studies reported in Deregt was there a mention of using heat shock proteins in any form to enhance the recombinant E2 antigens. The disclosures only related to the use of conventional adjuvants. In summary, in contrast to the disclosures in Deregt, the present application:

a) used a combination of antigenic proteins (e.g., at least two E2 proteins, or two E0 proteins and a single NS3 protein);
b) vaccinated animals against one strain of BVDV and then challenged them with another different strain (a far more severe test than any previously reported pestivirus antigen trial conducted in any animal);
c) provided a vaccine (comprising a hsps/antigenic polypeptide complex) that gives 100% protection against BVDV infection in the fetuses of vaccinated animals; and
d) provided a vaccine wherein the antigenicity of the antigenic protein is enhanced by coupling the antigen to an hsps.

None of these features were taught or suggested in Deregt.

As discussed above, Srivastava only explicitly teaches the use of mammalian hsps to form immunogenic hsps/antigen complexes. Deregt discusses only the E2 antigen as a vaccine (and does not teach the use of antigens coupled to any hsps at all). Therefore, even if a skilled person were to combine Srivastava and Deregt, they would be led to the generation of mammalian hsps coupled with just the E2 subunit pestivirus protein.

There would be no motivation for the skilled person to take the teachings of Deregt, which indicate that E2 is not an effective antigen for vaccination (and which does not suggest further proteins such as E0 and NS3 at all), and combine this with the teachings of Srivastava, which does not explicitly teach the use of non-mammalian hsps, to arrive at the method of producing the non-mammalian hsps/antigenic polypeptide immunogenic complexes provided in the present application.

For all of the foregoing reasons, Applicant submits that the Examiner has not established that each limitation of any of the rejected claims is taught or suggested, has not identified any motive or suggestion to modify the teachings of the references, and has not established a reasonable expectation of success. Accordingly, a *prima facie* case of

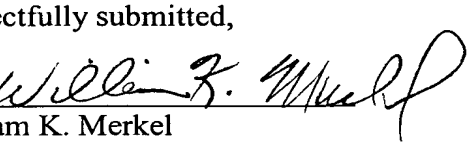
obviousness for any of claims 21, 23-24, or 29-31 under 35 U.S.C. § 103(a) over Srivastava has not been established and the rejection should be withdrawn.

VI. Conclusions

For all of the foregoing reasons, Applicant submits that each of the rejections of claims 21 and 23-31 has been overcome and the claims are now in condition for allowance. The Examiner is invited to contact the undersigned at the telephone number listed below in order to discuss any remaining issues or matters of form that will move this case to allowance.

Dated: March 13, 2006

Respectfully submitted,

By 

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Heat Shock Protein–Peptide Complexes, Reconstituted In Vitro, Elicit Peptide-specific Cytotoxic T Lymphocyte Response and Tumor Immunity

By Nathalie E. Blachere, Zihai Li, Rajiv Y. Chandawarkar, Ryuichiro Suto, Navdeep S. Jaikaria, Sreyashi Basu, Heiichiro Udono, and Pramod K. Srivastava

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Summary

Heat shock protein (HSP) preparations derived from cancer cells and virus-infected cells have been shown previously to elicit cancer-specific or virus-specific immunity. The immunogenicity of HSP preparations has been attributed to peptides associated with the HSPs. The studies reported here demonstrate that immunogenic HSP–peptide complexes can also be reconstituted in vitro. The studies show that (*a*) complexes of hsp70 or gp96 HSP molecules with a variety of synthetic peptides can be generated in vitro; (*b*) the binding of HSPs with peptides is specific in that a number of other proteins tested do not bind synthetic peptides under the conditions in which gp96 molecules do; (*c*) HSP–peptide complexes reconstituted in vitro are immunologically active, as tested by their ability to elicit antitumor immunity and specific $CD8^+$ cytolytic T lymphocyte response; and (*d*) synthetic peptides reconstituted in vitro with gp96 are capable of being taken up and re-presented by macrophage in the same manner as gp96–peptides complexes generated in vivo. These observations demonstrate that HSPs are $CD8^+$ T cell response–eliciting adjuvants.

Immunization with heat shock protein (HSP)[1] preparations isolated from cancer cells or virus-infected cells has been reported to elicit protective antitumor or antiviral cellular immune response (1–8). This paradigm has also been substantiated in other antigenic systems, such that gp96 HSP preparations isolated from a cell expressing a transfected cytosolic protein can immunize and elicit specific CTLs against that antigen (9). Similarly, gp96 preparations isolated from cells expressing a given set of minor H antigens can be used to immunize and elicit CTL response against the minor antigens expressed by the cells that were the source of the immunizing gp96 preparation (9). HSPs are not polymorphic molecules and do not differ in their primary structure among normal tissues and cancers, or among normal and virus-infected cells. In this light, the remarkably general immunizing ability of HSP preparations has been explained on the basis of the suggestion that the HSP molecules are associated with peptides generated in the cells from which the HSPs are isolated (10). Peptides associated with gp96 and hsp70 have since been demonstrated (6, 11) and it has been shown that dissociation of the HSP-bound peptides leads to abrogation of immunogenicity of the HSP preparation (6). Confirmation of these results has also been obtained in a viral system, as a recent study has demonstrated that gp96 preparations isolated from vesicular stomatitis virus (VSV)-infected cells contain VSV-derived peptides (12). It has been suggested that cytosolic and endoplasmic reticular HSPs chaperone antigenic peptides during antigen processing and presentation by MHC class I molecules (13). The mechanism by which such noncovalent HSP–peptide complexes elicit protective cellular immune responses has recently been elucidated (14, 15).

The HSP–peptide interaction is at the center of this newly emerging immunological paradigm. In this report, we demonstrate that HSP–peptide complexes can also be generated in vitro and that the biological activity of these complexes is comparable to that of HSP–peptide complexes generated in vivo. Further, the HSP–peptide complexes reconstituted in vitro elicit immunity by a mechanism apparently identical to that implicated in the immunogenicity of the complexes generated in vivo.

Materials and Methods

Mice and Cell Lines. Female C57BL/6 (6–8 wk old) were purchased from The Jackson Laboratory (Bar Harbor, ME). EL4 cells are a thymoma of C57BL/6 origin. N1 is a clone of EL4 trans-

[1] *Abbreviations used in this paper:* HSP, heat shock protein; NP, nucleocapsoid protein; VSV, vesicular stomatitis virus.

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fected with the nucleocapsid gene of VSV (16). VSV-specific CTLs were derived from mice that were immunized with irradiated (7,500 rads) N1 cells. 7 d after immunization, splenocytes (8×10^6) from immune mice were cultured with irradiated N1 cells (5×10^4) in 24-well plates. CTLs were restimulated every 7 d. Pristane-induced peritoneal exudate cells were used for macrophage enriched population.

Purification of HSPs. gp96 was purified from C57BL/6 liver cells, as described (2). In brief, 15 livers were homogenized in 40 ml of hypotonic buffer (30 mM $NaHCO_3$, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.1) by a tissue tearor, and a 100,000 *g* supernatant was obtained. The supernatant was fractionated by 50–70% ammonium sulfate precipitation, applied to a concanavalin A–agarose column, and glycoproteins were eluted by 10% α-methylmannoside. The eluate was applied to a DEAE–agarose column, equilibrated with 0.3 M NaCl, and was eluted with 0.7 M NaCl. Hsp70 was purified as described by Peng et al. (17).

HSP–Peptide Binding. gp96 and ^{125}I-labeled peptides (synthesized by Bio-Synthesis, Inc., Lewisville, TX), were mixed in the quantities indicated, and incubated for 10 min at the indicated temperatures in a binding buffer (20 mM Hepes, pH 7.2, 20 mM NaCl, and 2 mM $MgCl_2$). The samples were then incubated for 30 min at room temperature. Alternatively, gp96 and peptides were coincubated in sodium phosphate buffer at 25 or 50°C, as indicated, for 10 min at various salt concentrations, followed by incubation at room temperature for 30 min. In the case of hsp70, high temperatures and high salt concentrations were unnecessary; hsp70 and peptides were coincubated at 37°C in sodium phosphate buffer containing 1 mM ADP and 1 mM $MgCl_2$. Free peptide was removed completely using a microcon 50 (Amicon, Inc., Beverly, MA). The removal of free peptides was monitored by electrophoretic analysis of the labeling mixture, followed by quantitative autoradiography; if peptides were not removed, they were visible on the dye front. Samples were also analyzed by silver staining or immunoblotting with anti-gp96 antibody (anti-GRP94, SPA-850, clone 9G10; NeoMarkers, Fremont, CA) or anti-hsp70 antibody (clone BRM22 from NeoMarkers). Peptide quantification was determined by densitometry using the Quantity 1 (version 2.2) program with the PDI Discovery series system (Sun Microsystems).

Tumor Rejection Assay. Mice were injected subcutaneously with 10 μg or 25 μg reconstituted HSP–peptide complexes, or gp96 alone, peptide alone (75 nM), or buffer twice at weekly intervals. Mice were challenged intraperitoneally with 5,000 live N1 tumor cells 7 d after the second immunization.

CTL Assay. Spleen cells (8×10^6/well) from immunized mice (day 96) were cultured in mixed lymphocyte tumor culture with 7,500 rads irradiated antigen-positive cells or cognate peptide-pulsed cell (5×10^4/well) in 24-well plates. After 5 d, mixed lymphocyte tumor cultures were tested for cytotoxicity in a chromium release assay.

TNF-α Bioassay. Macrophages (1.5×10^4) and VSV-specific CTLs (5×10^4) were cultured with serially diluted reconstituted VSV–gp96 complexes for 24 h at 37°C. Supernatants were collected and assayed for TNF-α production in a cytotoxicity assay as described (15).

Results

Exchange of Peptides Naturally Bound to HSPs with Exogenous Peptides. The ability of gp96 molecules to bind peptides in vitro was analyzed using an electrophoretic assay. The rationale for the use of this assay was as follows: it had been demonstrated earlier that gp96 preparations obtained from preparative sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) could still be used to elicit limited but significant tumor-specific immunity (2). This observation seen in the context of subsequent studies, which suggested that gp96 preparations are immunogenic because of association of gp96 with antigenic peptides (11, 12), indicated that gp96–peptide interaction would be expected to be stable under conditions of SDS-PAGE.

The peptide A (KRQIYTDLEMNRLGK) derived from G protein of the VSV was used for the initial studies. This peptide has been shown previously to bind hsp70 molecules in vitro (18). Apparently homogeneous, unlabeled gp96 preparations were incubated at 37°C with iodinated peptide A as described in Materials and Methods. The sample was analyzed by SDS-PAGE with or without additional heating of the sample in SDS-PAGE sample buffer, followed by autoradiography. The expectation from such an experiment is that SDS-resistant binding of unlabeled gp96 to labeled peptide will result in a labeled 96-kD band. However, no binding of gp96 to peptide A is detected under these conditions. The possibility was considered that incubation of gp96 with exogenous peptides at higher temperatures might permit dissociation of naturally bound peptides followed by reannealing of a proportion of exogenously added radiolabeled peptides at lower temperatures. Iodinated preparations of peptide A were incubated with unlabeled gp96 at 4, 25, 37, 60, or 90°C for 10 min and allowed to cool to room temperature for an additional 30 min. The samples were analyzed by SDS-PAGE without further heating and the gels were stained for proteins and autoradiographed. It was observed (Fig. 1) that exogenously added labeled peptide A could associate with gp96 in a temperature-dependent manner with optimal binding at 60°C. Little binding is detected at 4, 25, 37, or 90°C. Although the intensity of label in the gp96 band varies at different temperatures and at different peptide concentrations (Fig. 1, *A* and *B*), the quantity of gp96 as detected by silver staining is constant in all lanes (Fig. 1 *C*). It was also observed that the gp96–peptide binding can be dissociated, if the complexes are heated in a boiling water bath (data not shown).

The exchange of exogenous and native-bound peptides could also be achieved by incubation of gp96 with exogenous peptides at high salt concentrations. Gp96 preparations were incubated at 25 or 50°C with radio-iodinated peptide VSV19 (extended on both termini of K^b-binding VSV nucleocapsid protein (NP)-derived octamer VSV8) for 10 min in sodium phosphate buffer containing 200 mM, 300 mM, 500 mM, 700 mM, 800 mM, 1 M, 2 M, or 3 M NaCl, followed by 30 min at room temperature. The samples were desalted and analyzed by SDS-PAGE, followed by staining as well as autoradiography. It was observed (Fig. 2) that significant quantities of labeled peptides formed an SDS-resistant association with gp96 after incubation at 2 M or higher NaCl concentration, but not at lower concentrations. In presence of high salt, the extent of

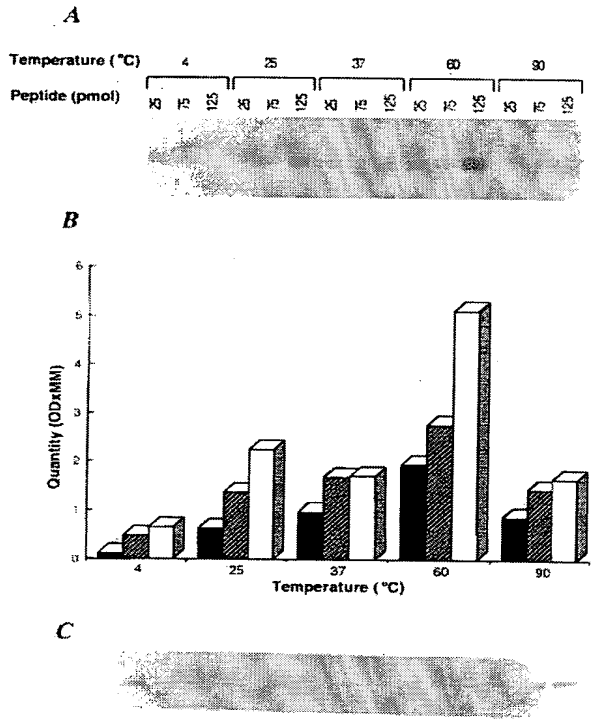

Figure 1. gp96 binds to peptides in vitro. gp96 (10 pmol) was incubated with increasing concentrations of radioiodinated peptide A (25, 75, and 125 pmol) for 10 min at different temperatures in 20 μl reaction buffer, followed by 30 min at room temperature. The reaction was terminated by mixing with sample buffer (0.1% SDS, 20% glycerol, and 5% bromophenol blue) and analyzed by SDS-PAGE. (*A*) Autoradiogram after 48-h exposure. (*B*) Densitometric quantification of results in *A*. An aliquot of each reaction was analyzed in parallel by SDS-PAGE and silver staining (*C*).

association of gp96 with peptides was comparable at the low and high temperatures, whereas at low salt concentrations, gp96–peptide interaction was detected only at the higher temperature. The quantity of gp96 in each lane, as judged by Coomassie blue staining and scanning, was identical.

Reconstitution of peptides with hsp70 molecules was observed to require neither a heating and cooling cycle, nor exposure to high salt concentrations. Incubation of apparently homogeneous preparations of hsp70 with radiolabeled peptide A in sodium phosphate buffer containing 1 mM ADP and 1 mM $MgCl_2$ at 37°C was found to be sufficient to generate SDS-stable hsp70–peptide complexes as judged by autoradiography (see Fig. 5 *A*).

Binding of gp96 or hsp70 to peptides is not restricted to peptide A and can also be demonstrated for an array of other peptides, such as peptide B, LSSLFRPKRRPIYKS (derived from VSV G protein; reference 18); peptide C, SLSDLRGYVYQGLKSGNVS (derived from VSV nucleoprotein; reference 18); peptide D, IASNENMETMESSTLE (derived from nucleoprotein of influenza virus strain

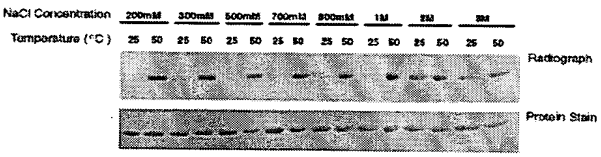

Figure 2. Exchange of exogenous and native-bound peptides at high salt concentrations. gp96 (40 pM) and iodinated synthetic peptide (2 nM, NH_2–Ser–Leu–Ser–Asp–Leu–Arg–Gly–Tyr–Val–Tyr–Gln–Gly–Leu–Lys–Ser–Gly–Asn–Val–Ser–COOH) were mixed in phosphate buffer in 20 μl reaction volume and incubated at 25 or 50°C for 10 min. After centrifugation, the mixtures were incubated at 25°C for another 30 min. Samples were analyzed by SDS-PAGE and staining, followed by autoradiography of the stained gel (24-h exposure).

A/PR/8/34); and peptide E, SFIRGTKVSPRGKLST (derived from nucleoprotein of influenza virus A/NY/60/68) (data not shown). To evaluate the specificity of binding of peptides to gp96, unlabeled peptides A, B, C, D, and E were tested for their ability to compete with labeled peptide A in the gp96–peptide A binding assay. gp96, 25 pmol radiolabeled peptide A, and 0.1 or 10 nmol unlabeled peptides A, B, C, D, or E were coincubated at 50°C, followed by a 30-min incubation at room temperature. It was observed that all peptides could compete with peptide A in binding to gp96, although with different efficiencies (Fig. 3 *A*). As expected, higher quantities (10 nmol) of competing unlabeled peptides were more effective in displacing labeled peptide A than the lower quantities in the case of all peptides except peptide E, in which case the competition was already saturating at the lower quantity.

The specificity of binding of HSPs with the exogenous peptide was demonstrated in the following additional ways, as shown here for gp96, but also observed for hsp70. (*a*) Inclusion of BSA or OVA in gp96–peptide A binding reaction had no influence on gp96–peptide A binding (Fig. 3 *B*); (*b*) a number of proteins, i.e., α–2 macroglobulin, β-galactosidase, fructose-6-phosphate kinase, OVA, pyruvate kinase, fumarase, and triosephosphate isomerase were tested for their ability to bind peptide A and were observed to not bind it (Fig. 3 *C*); and (*c*) it was demonstrated that only the intact gp96 and not any of its various degradation products could bind peptide A (Fig. 3 *C*).

The quantity of peptide bound to the HSPs in vitro was determined. The specific radioactivity of the peptides (cpm/mol of peptide) was measured; using this number, the number of moles of peptides bound to a given quantity of gp96 were determined by measuring the cpm in the HSP band after autoradiography, by cutting out the band and counting it in a γ counter. This calculation revealed that under the conditions tested, and assuming a stoichiometry of one peptide per HSP molecule, ~1% of HSP molecules were loaded with the exogenous peptide. The assumption of a 1:1 stoichiometry between HSP and peptides was made on the basis of the recent demonstration of a single peptide-binding pocket in a bacterial hsp70 molecule (19).

Immunogenicity of HSP–peptide Complexes Reconstituted In Vitro. gp96–peptide complexes and hsp70–peptide com-

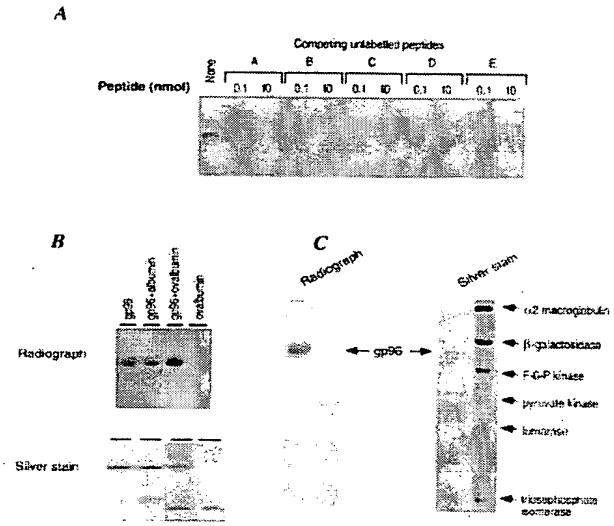

Figure 3. Specificity of peptide binding by gp96. (*A*) Unlabeled peptides *A*, *B*, *C*, *D*, and *E* (0.1 and 10 nM) were used to compete with labeled peptide *A* (25 pmol) for the binding to gp96 (10 pmol). The sequences of these peptides are described in the text. The binding assay described in the legend to Fig. 1 was used, except that binding was carried out at 50°C. (*B*) Albumin and ovalbumin do not compete with gp96 for binding to peptide A. gp96 (10 pmol) was incubated with 25-pmol radiolabeled peptide A and analyzed as in the legend to Fig. 1. Albumin and ovalbumin (10 pmol each) were included in the binding assay. The autoradiogram and the silver stained gel are shown. (*C*) A partially degraded preparation of gp96 and a mixture of six purified proteins (i.e., α–2 macroglobulin, β-galactosidase, fructose-6-phosphokinase, pyruvate kinase, fumarase, and triosephosphate isomerase) were tested for binding to iodinated peptide *A*. Only the intact gp96 molecule is able to form a stable complex with radioactive peptide *A*. None of the other six proteins tested are able to bind peptide *A*.

plexes generated in vitro were tested in a variety of models for their ability to elicit CTLs and tumor immunity. For generation of CTLs, seven model peptides, which bind to different MHC class I alleles, were tested. These derive from OVA (K^b), SV40 T antigen (D^b), NP antigen of influenza virus (D^b and K^b), NP antigen of VSV (K^b), and β-galactosidase (L^d). The peptides were complexed with gp96, hsp70, or both and mice of the appropriate haplotype (b or d) were immunized twice at weekly intervals, with the peptides alone (10 μg peptide in PBS), the uncomplexed HSPs alone (20–50 μg HSP in PBS), or the HSP–peptide complexes (20–50 μg HSP complexed with ~2 ng peptide, determined as described in the previous section). Spleen cells from the immunized mice were put in culture and were stimulated with the cognate peptide and tested for cytotoxic activity on target cells pulsed or unpulsed with relevant peptides. It was observed (Fig. 4) that T cells obtained from mice immunized with peptides alone or with gp96 or hsp70 alone showed no cytotoxic activity, whereas T cells obtained from mice immunized with HSP–peptide complexes showed significant and consistent peptide-specific CTL activity. The precise MHC class I–binding peptides were used in these studies. To test whether larger peptides than the exact epitopes could be complexed with HSPs and used to immunize successfully, a 19-mer precursor of K^b-binding VSV8 (VSV19) was complexed with hsp70 and gp96 and the complexes used to immunize. The HSP–VSV19 complexes were found to be as effective at eliciting antigen-specific CTL response as the HSP–VSV8 complexes were (Fig. 4).

A number of parameters of the adjuvant activity of gp96 and hsp70 were tested. The possibility that complexing of HSPs with peptides is unnecessary and HSP–peptide mixtures (instead of complexes) may be equally immunogenic was tested. It was observed that, similar to the results of immunization with HSPs or peptides alone, mixtures of HSPs and peptides were consistently nonimmunogenic. Similarly, when mice were immunized with HSPs on one flank and peptides on the other, no CTL response was detected (data not shown).

The possibility that immunization with peptides mixed or complexed with any large molecule, particularly other carrier molecules, might also elicit a potent and specific CTL response was investigated. The VSV9 peptide (Tyr–VSV8) was mixed with a traditional carrier protein, mouse serum albumin, under conditions that facilitate binding with gp96 and hsp70. The complexed material was analyzed by SDS-PAGE, and mouse serum albumin, like gp96 and hsp70, was found to form an SDS-resistant complex with the peptide (Fig. 5 *A*). Mice were immunized with mouse serum albumin–VSV9 complex, and were tested for CTL response as described previously. No CTL response was detected (Fig. 5 *B*). On the other hand, Gp96–VSV9 and hsp70–VSV9 complexes elicited significant specific CTL responses (Fig. 5 *B*).

These observations demonstrate that the HSPs gp96 and hsp70 possess an adjuvant activity effective for eliciting a $CD8^+$ T cell response. The efficacy of HSP–peptide vaccination in eliciting tumor rejection was tested in an artificial model, because the identity of peptides that can elicit rejection of natural tumors is yet unknown. Tumor rejection studies were carried out using the N1 tumor, which has been derived by transfection of the EL4 lymphoma of the C57BL/6 mouse with the gene encoding the NP of the VSV. Therefore, VSV–NP is a model tumor rejection antigen for this tumor (16). (In the past, we have actively refrained from using tumor models in which foreign genes have been transfected into tumors, as they reveal little about tumor immunity. However, this model is used in the present study to measure an antigen-specific T cell response in vivo.) Thus, gp96 molecules obtained from normal livers of C57BL/6 mice were complexed with the VSV8 known to bind K^b. C57BL/6 mice were immunized with such reconstituted complexes, or with VSV8 alone, or with liver gp96 alone, and were challenged with N1 cells. Survival of mice was monitored (Fig. 6). It was observed that there was no difference in the survival kinetics of unimmunized mice and mice immunized with liver gp96 or the VSV8 alone: all mice died within 30–50 d of tumor challenge. In contrast, 8 of 10 mice immunized with gp96–VSV8 complexes

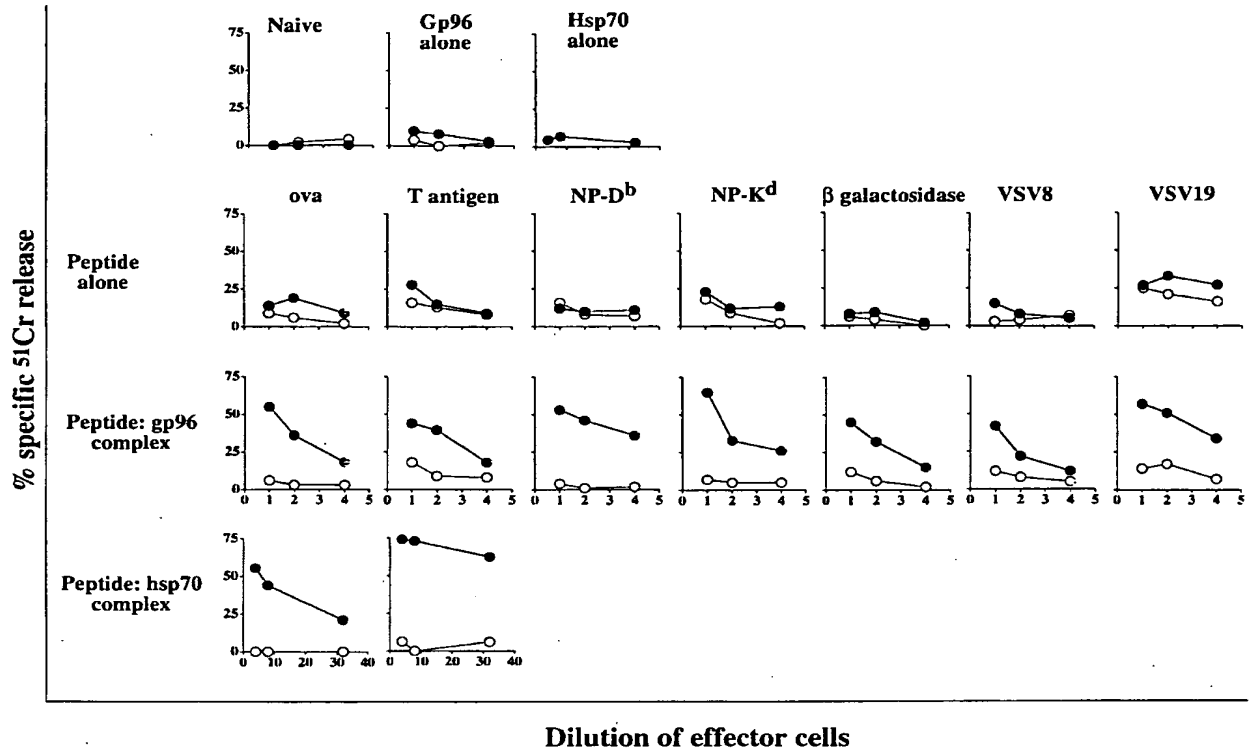

Figure 4. Hsp–peptide complexes reconstituted in vitro prime mice for CD8$^+$ T cell response. Mice were immunized with HSPs alone (20–50 μg), peptides alone (10 μg), or HSP–peptide complexes (20–50 μg), as indicated. 1 wk after the last immunization, spleens were removed and stimulated with the cognate peptide or with cells transfected with the gene encoding the relevant antigen. The lymphocyte cultures were tested for their ability to lyse cells transfected with the antigen of interest (*closed circle*) and the nontransfected parental line (*open circle*). For the top panel, HSPs alone were tested for their immunizing ability in each antigenic system and were found to be consistently negative. The CTL responses were tested in many but not all systems and where tested, were found to be MHC class I and CD8 restricted.

reconstituted in vitro, survived beyond 100 d after tumor challenge. Spleens of the immunized mice were also tested for antigen-specific CTL response to the VSV8 epitope. It was observed that mice immunized with the gp96–VSV8 complex generated effective antigen-specific, CD8$^+$ CTL response, whereas mice immunized with gp96 alone, or the VSV8 alone, did not (data not shown). These results indicate that the peptides complexed with gp96 in vitro elicit tumor immunity in a manner consistent with the gp96–peptide complexes generated in vivo. Similar antitumor activity has been shown for hsp70–peptide complexes (data not shown).

Re-presentation of Peptides Reconstituted with gp96 In Vitro, by MHC Class I Molecules of Macrophages. The mechanism whereby immunization with gp96–peptide complexes generated in vivo leads to a protective CTL response has been elucidated (15). It has been shown that gp96–peptide complexes are taken up by macrophages and the chaperoned peptides are re-presented by the MHC class I molecules of the macrophage through a novel pathway. The immunological activity of the gp96–peptide complexes generated in vitro was tested in this assay. gp96 preparations were reconstituted with the VSV8 peptide at different temperatures and the resulting complexes were used to pulse pristane-induced macrophages of C57BL/6 mice in vitro. The pulsed macrophages were tested for their ability to stimulate anti-VSV CTLs, as measured by the secretion of TNF-α by the CTLs (Fig. 7). It was observed that the macrophage pulsed with complexes reconstituted at 60°C were effective in this re-presentation assay, whereas those reconstituted at 37, 80, or 98°C were not. As we have shown previously (15), the quantity of VSV8 complexed with gp96 in these experiments is ~2 log scales lower than that necessary for direct charging of the empty surface MHC class I molecules by the VSV8 peptides. Data in Fig. 7 show that the gp96–peptide complexes reconstituted in vitro appear to be re-presented by the antigen-presenting cells in the same manner as shown previously (15) for the natural HSP–peptide complexes.

Discussion

The studies described here indicate that HSP–peptide complexes can be reconstituted in vitro and that, by all parame-

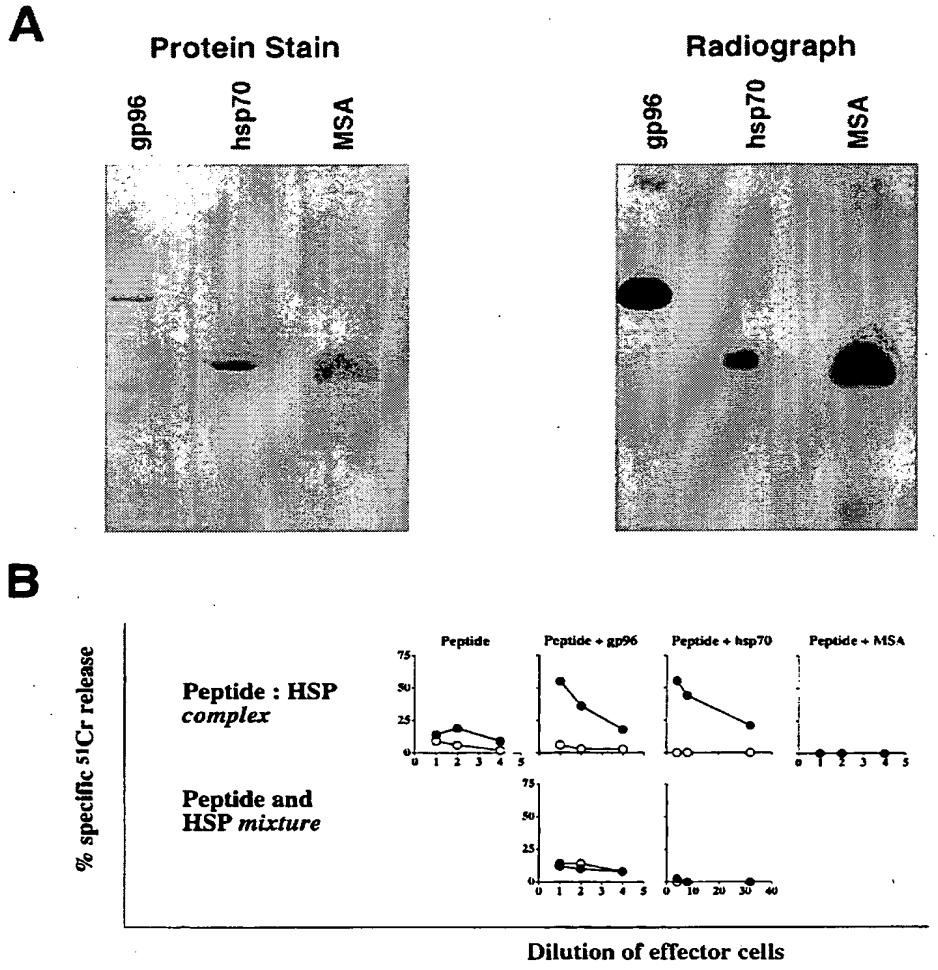

Figure 5. Chaperoning of peptides by HSPs is required for generation of an effective $CD8^+$ T cell response. gp96, hsp70, or mouse serum albumin (*MSA*) were complexed with radiolabeled VSV9 and analyzed by (*A*) SDS-PAGE followed by Coomassie blue staining and autoradiography. In addition, mice were immunized (*B*) with peptides complexed or simply mixed with each of the proteins. Splenocytes of these mice were tested for induction of $CD8^+$ T lymphocytes, as described in legend to Fig. 4. N1 (*closed circle*) and EL4 (*open circle*) were used as targets.

ters tested, such complexes show immunological activity similar to the HSP–peptide complexes generated in vivo. The results also show significant differences between gp96 and hsp70 with respect to the conditions in vitro, under which they bind peptides. These differences presumably reflect the fact that although hsp70–ATP interaction plays a crucial role in hsp70–peptide interaction in vivo, the identity of the corresponding ligand for gp96 is presently unknown. In contrast with the situation with hsp70, gp96–ATP interaction does not strip gp96 of its associated peptides (data not shown), even though gp96, like hsp70, is an ATP-binding protein and is an ATPase (11). Exposure to high temperature and high salt apparently causes the gp96 molecule to assume an open conformation, which permits dissociation from and association with exogenous peptides. The identity of the ligands that catalyze this process in vivo would be of interest in this regard.

The observations reported here have several implications. First, they support the hypothesis that immunogenicity of tumor-derived gp96 preparations results from a physical association of gp96 with antigenic peptides. The HSP–peptide complex elicits immunity under conditions in which the HSP molecules alone, or the peptides alone, do not. Second, these observations show that one does not have to rely on HSP–peptide complexes generated in vivo to elicit immunity; instead, such complexes can be generated reproducibly in vitro, provided the identity of the immunogenic peptides is known. A variety of peptides of different lengths, compositions, and hydrophobicity can bind the HSPs, suggesting that the nature of an epitope is not a limiting factor in its suitability as a vaccine in the form of a HSP–peptide complex. The ability of the gp96 to bind peptides in vitro has also been independently demonstrated recently (20). The quantity of peptide that is required to be conjugated to the HSPs is extremely small and 1–2 ng of peptides complexed to the HSPs elicit potent cellular immune response. At first sight, this quantity may appear to be unrealistically small; however, when it is considered that the peptides chaperoned by the HSPs are targeted specifically to the professional antigen-presenting cells (15, 21), 1–2 ng or ~6

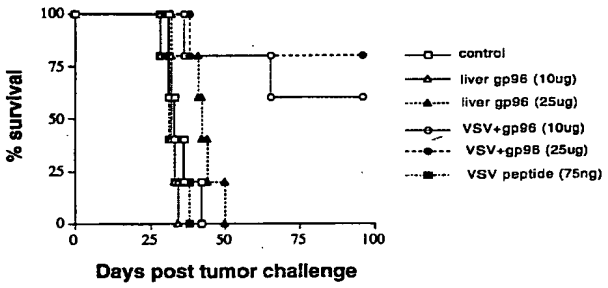

Figure 6. gp96–VSV8 complexes reconstituted in vitro elicit peptide-specific, protective immunity. Mice were immunized twice at weekly intervals with gp96–VSV8 complexes (10 μg or 25 μg liver gp96 complexed with 1–2 ng VSV8 peptide), liver gp96 alone (10 or 25 μg), VSV8 peptide alone (75 ng), or RPMI medium control. gp96–VSV8 complexes were washed extensively using a minicon 50 to remove unbound peptides. All mice were challenged intraperitoneally with 5,000 live N1 cells 1 wk after the second immunization; survival was monitored.

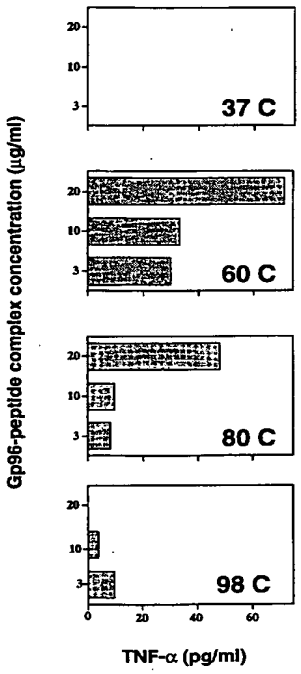

Figure 7. VSV8 peptide chaperoned by gp96 is re-presented by macrophage. Pristane-induced peritoneal exudate cells (10^4) and VSV peptide-specific CTL (5×10^4) were cocultured with gp96–VSV8 complexes (3–20 μg/ml) reconstituted at 37, 60, 85, or 98°C in a 96-well U-bottomed plate at 37°C. After 24 h, supernatants were collected and assayed for TNF-α production in a cytotoxicity assay as described (15).

$\times 10^{11}$ molecules of specific peptide targeted to the relevant antigen-presenting cells are actually a large number, as argued in more detail elsewhere (22). This observation has significant implications for vaccination against infectious diseases in which the protective epitopes are known, and for any cancers, such as those of viral etiology, that may share antigenic epitopes.

Essentially, these results show that HSPs are adjuvants. This adjuvanticity has a number of unique characteristics: in contrast with other nonlive adjuvants, the adjuvanticity of HSPs generates MHC class I–restricted T cell responses. No serological antipeptide response has ever been detected among the tens of immunized mice tested (data not shown). The quantitative requirements of antigens administered with HSPs are log scales lower than corresponding requirements for other adjuvants. Finally, HSPs are the first adjuvants of mammalian origin. We have suggested previously that the immunogenicity of HSP–peptide complexes may reflect the role in vivo of such complexes in priming of cellular immune responses (23). In this view, the observed adjuvanticity of HSPs is simply a reflection of the natural role of HSPs in vivo.

The structural basis of the ability of gp96 molecules to bind a variety of peptides is presently unclear and requires further study. Obviously, there are certain rules for the HSP–peptide interaction as seen in the observation that peptides differ in their ability to compete with a given peptide for binding to gp96 (Fig. 3). However, the studies carried out here are not of a broad enough scope to permit elucidation of these rules. Broadly speaking, HSP–peptide interaction is reminiscent of MHC–peptide interaction, which was equally mysterious as to its structural basis until the rules of interaction were identified (24). The MHC and the HSPs share a number of crucial properties, such as the ability to bind peptides, a ubiquitous tissue distribution, high degree of phylogenetic conservation, inducibility of the respective genes by IFN-γ (25) and, finally, the ability to prime CTL responses against the peptides chaperoned by them. These considerations led us in the past (26) to suggest a phylogenetic relationship between the MHC and the HSPs, and a number of recent observations (19, 27–28) have not been inconsistent with that suggestion. The association of peptides with HSPs of the cytosol (hsp70 and hsp90) and the endoplasmic reticulum (gp96) had also led us to suggest that HSPs constitute a relay line of molecules that chaperones the peptides and ultimately delivers them to the MHC class I molecules (23). Therefore, the HSPs were suggested to be accessories to antigen presentation by MHC class I molecules. Our recent results, which show that peptides precursors to the MHC class I–binding epitopes are found in specific association with hsp70, hsp90, and gp96 (Ishii et al., manuscript submitted for publication), are in accord with our suggestion. The recent demonstration by Lammert et al. (29) that the HSP gp96 acts as a major peptide acceptor for peptides transported into the lumen of the endoplasmic reticulum through transport-associated protein molecules, also supports the relay-line hypothesis.

N.E. Blachere and Z. Li contributed equally to this work and are listed in alphabetical order.

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Isolation of MHC Class I-Restricted Tumor Antigen Peptide and Its Precursors Associated with Heat Shock Proteins hsp70, hsp90, and gp96[1]

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We have previously demonstrated that vaccination with heat shock proteins hsp70, hsp90, and gp96 elicits specific immunity against the tumor from which the hsps were purified. Although the association of tumor Ag peptides with these hsps have been suggested, the identification of the peptides or their precursors stripped from the hsps remained to be resolved. We show in this report that an L^d-restricted cytotoxic T lymphocyte epitope of a mouse leukemia RL♂1 and its precursors are associated with the chaperones hsp90 and hsp70 in the cytosol and gp96 in the lumen of the endoplasmic reticulum. Hsp70 was associated with only final sized octamer, while hsp90 was found to associate with the octamer and two distinct precursor peptides. The gp96 was associated with the octamer and one of the two precursors. Thus, each of the hsps bound a distinct set of peptides. Our results have demonstrated for the first time that the hsps associate not only with final sized tumor Ag peptide but also with its precursors. The implication of this evidence is also discussed in terms of the roles of hsps in MHC class I Ag processing/presentation. *The Journal of Immunology,* **1999, 162: 1303–1309.**

It has been demonstrated that vaccination with heat shock proteins hsp70,[3] hsp90, and gp96, derived from cancer tissues but not from normal tissues, renders mice resistant to the tumors from which the hsps were isolated (1–3). Previously we suggested that this specific immunity is caused by tumor Ag peptides chaperoned by these hsps, and the peptides are channeled into the MHC class I processing pathway in APCs (4–6). Indeed, hsps isolated from virus-infected cells elicit virus-specific $CD8^+$ CTLs (7–9); gp96 isolated from cells transfected with a gene encoding a model Ag elicits CTLs against the Ag (10). Structural support for the immunological data was obtained when Nieland et al. showed that a K^b-binding VSV nucleocapsid peptide epitope was associated with gp96 purified from VSV-infected cells (11). Consistent with the lack of polymorphism of hsps and with the ability of hsps preparations to cross-prime across H-2 haplotypes, the association of the K^b-binding epitope with gp96 was demonstrated in VSV-infected cells of the H-2^k or the H-2^d haplotype. In this study, however, the association of cytosolic hsp70 or hsp90 with the final epitope or its precursors was not addressed. Recently, Breloer et al. identified OVA-derived K^b-binding peptide associated with hsp70 and gp96 (12), which were isolated from the OVA-transfected cell line E.G7, although the association of these hsps with the precursors was not observed. Further, the association of hsp90 with the endogenously produced peptides has not been demonstrated yet in any experimental systems.

In the tumor immunology, the original thesis that the immune response was elicited by tumor Ag peptides associated with hsps but not by hsps themselves remained uncertain because the real entity of the peptides associated with hsps that cause the tumor regression was not known; that is, whether it is possible to purify exact CTL epitopes or their precursors from hsps that could sensitize targets for lysis by tumor-specific CTLs. To answer this question as well as to show the generality of the observations of Nieland et al. (11) and Breloer et al., we examined here a tumor system that demonstrates association of an L^d-restricted peptide (IPGLPLSL, or pRL1a) and its longer precursors with the three hsps, hsp70, hsp90, and gp96, of the BALBRL♂1. The pRL1a epitope is derived from the 5′ untranslated region of the *akt* gene (13, 14) expressed in this leukemia and acts as a tumor rejection Ag for this leukemia (15).

Materials and Methods

Animals and cell lines

RL♂1 and Meth A cells were expanded in ascitic form in BALB/c mice. The mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and were bred in pathogen-free condition at the animal facility of Okayama University Medical School (Okayama, Japan). RL♂1-specific CTL clone Y-15 was maintained by weekly stimulation with irradiated RL♂1 cells and feeder cells as described previously (16).

Purification of gp96, hsp90, hsp70, and L^d molecules

The gp96 and hsp90 were purified simultaneously as described previously (17). Briefly, a 10-ml cell pellet ($\sim 10^{10}$ cells) of ascitic RL♂1 cells of BALB/c mice was homogenized in 40 ml of a hypotonic buffer (30 mM $NaHCO_3$, and 0.5 mM PMSF (pH 7.1)), and its 100,000 × *g* supernatant was applied to a Con A-Sepharose (Pharmacia Biotech, Uppsala, Sweden) column followed by elution with 10% α-methyl-mannopyranoside. For isolation of gp96, Con A-bound material was resolved on a Mono Q FPLC

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[3] Abbreviations used in this paper: hsp(s), heat shock protein(s); gp, glycoprotein; FPLC, fast protein liquid chromatography; TFA, trifluoroacetic acid; ER, endoplasmic reticulum; grp, glucose-regulated protein; VSV, vesicular stomatitis virus.

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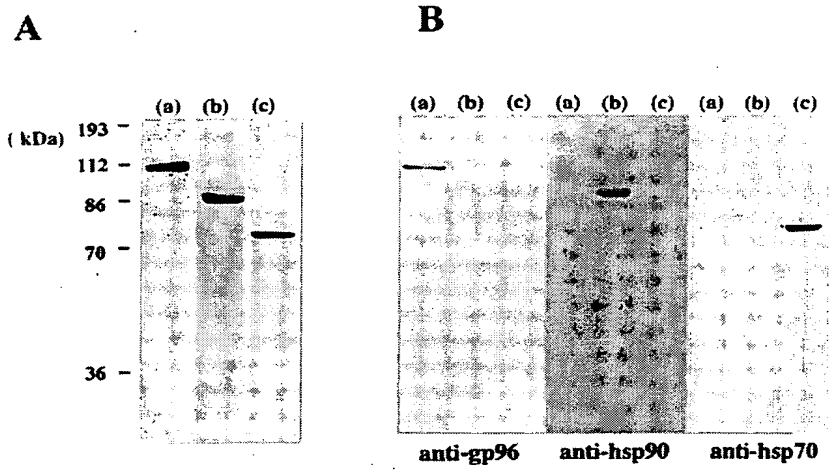

FIGURE 1. Homogeneous preparations of hsps gp96, hsp90, and hsp70 derived from RL♂1 cells. *A*, SDS-PAGE followed by silver staining of gp96 (*lane a*), hsp90 (*lane b*), and hsp70 (*lane c*) purified from RL♂1 cells (as described in *Materials and Methods*). *B*, Each of the three preparations, gp96 (*lane a*), hsp90 (*lane b*), and hsp70 (*lane c*), were immunoblotted with Abs against each, as indicated, to detect cross-contamination of one hsps with another; no cross-contamination was detected.

(Pharmacia) system equilibrated with 5 mM phosphate buffer (pH 7.0) and eluted by a 0–1 M NaCl gradient. For purification of hsp90, the Con A-unbound material was dialyzed against 20 mM of sodium phosphate (pH 7.4), 1 mM EDTA, and 250 mM NaCl and then resolved on the Mono Q system equilibrated with 20 mM sodium phosphate (pH 7.4), 1 mM EDTA, and 200 mM NaCl. The proteins were eluted by a 200–600 mM NaCl gradient. Hsp70 was purified as described previously (18, 19) with some modifications. For purification of hsp70, a 100,000 × *g* supernatant of the homogenate was dialyzed against buffer D (20 mM Tris acetate (pH 7.5), 20 mM NaCl, 0.1 mM EDTA, 15 mM 2-ME, and 3 mM $MgCl_2$) with a PD10 column (Pharmacia Biotech) and loaded onto an ADP-agarose (Sigma, St. Louis, MO) column. The column was extensively washed with 5 gel vol of buffer D plus 500 mM NaCl and with 2 vol of buffer D. Bound material was eluted with 3 mM ADP in buffer D and dialyzed with a PD10 column to FPLC buffer, then resolved on a Mono Q FPLC system equilibrated with 20 mM Tris acetate (pH 7.5), 20 mM NaCl, 0.1 mM EDTA, and 15 mM 2-ME. The proteins were eluted by a 20–500 mM NaCl gradient. Otherwise, the eluate was dialyzed against buffer E (20 mM sodium phosphate and 20 mM NaCl (pH 7.0)), resolved on a DEAE-Sepharose column (Sigma), and eluted by buffer E plus 130 mM NaCl. After this, fractions (1 ml) eluted by the Mono Q system or on a DEAE-Sepharose column were probed by SDS-PAGE followed by silver staining (Silver Stain Plus, Bio-Rad, Hercules, CA), and the fractions containing single bands of each hsps were used in the experiments. By these procedure approximately 1.5–2 mg of gp96, 2–2.5 mg of hsp70, and 4–6 mg of hsp90 were recovered from 1×10^{10} RL♂1 cells, respectively. These proteins were resolved on SDS-PAGE, blotted to nitrocellulose, and probed with anti-grp94 mAb (SPA-850, StressGen, Victoria, British Columbia), anti-hsp90 mAb (3G3, Affinity BioReagents, Golden, CO), and anti-hsp70 mAb (SPA-820, clone N27F3–4, StressGen), and alkaline phosphatase-labeled second Ab (Bio-Rad) was used for visualization. L^d molecules were purified from Nonidet P-40-treated RL♂1 cell lysate with an immunoaffinity column as described previously (13).

Dissociation of the endogenous peptides from gp96, hsp90, hsp70, and L^d

Approximately 2 mg of the purified hsp70 preparations in a 1-ml volume were exposed to 10 mM ATP and 3 mM $MgCl_2$ at 37°C for 1 h. Purified gp96 and hsp90 preparations (2 mg each) and immunoaffinity-purified L^d molecules were exposed to 0.2% trifluoroacetic acid (TFA) at 4°C for 1 h to strip peptides followed by centrifugation through an assembly with a molecular cut-off membrane (m.w. 10,000; Centricon 10, Amicon, Beverly, MA). The low m.w. fractions were pooled, concentrated by evaporation with a Speed-Vac (Savant, Farmingdale, NY), and dissolved in 0.1% TFA. These materials were applied to a C_{18} reverse phase HPLC column (Vydac, Hesperia, CA) pre-equilibrated with 0.1% TFA and water, and the bound materials were eluted at a flow rate of 0.5 ml/min. Solvent A was 0.1% TFA and water, and solvent B was 79.9% acetonitrile, 0.1% TFA, and 20% water. The gradient for chromatography was 20–60% B over 60 min, and fractions at 1-min intervals were collected. To avoid the cross-contamination of peptides between synthetic peptides and naturally processed peptides, we purchased two new C_{18} column; one column was used only for the separation of naturally processed peptides stripped from hsps, and the another was used for separation of synthetic peptides.

Cytotoxicity assay

The fractions collected by reverse phase HPLC were completely evaporated by the Speed-Vac and dissolved in 150 μl of 0.1% TFA. P-815 target cells were labeled by incubating 2×10^6 cells with 2 MBq of $Na_2{}^{51}CrO_4$ in 0.3 ml of RPMI (with 10% FCS) for 1 h at 37°C under 5% CO_2 in air. The cells were washed and cultured in 100 μl of medium at 5×10^3 cells/well in a 96-well round-bottom plate with sensitization by the peptides containing each 10 μl of each HPLC fractions for 1 h. Then the RL♂1-specific CTL clone Y-15 cells were added and cultured for 4 h. Finally the supernatants (100 μl) were removed, and their radioactivities were measured. The percent specific ^{51}Cr release was calculated by the following equation: $[(a - b)/(c - b)] \times 100$, where *a* is the radioactivity in the supernatant of target cells mixed with effector cells, *b* is the spontaneous release, and *c* is the maximum release after lysis of target cells with 1% Nonidet P-40.

Peptide synthesis

Peptides were synthesized by standard solid phase methods using F-moc chemistry in a peptide synthesizer (model 430A, Applied Biosystems, Foster City, CA). The peptides were purified by reverse phase HPLC on a preparative C_8 column (10 × 100 mm, 20-mm particle size; Applied Biosystems) in 0.1% TFA with an acetonitrile gradient and freeze-dried for stock.

Mass spectrometry analysis

The masses of the peptides were determined on-line by a tandem quadrupole mass spectrometer (TSQ 700, Finnigan MAT, San Jose, CA) equipped with an electrospray ion source. The peptides were identified by their molecular mass as the *m/z* peaks of single charged ion.

Competition assay by pRL1a and pRL1b of complex formation between hsp70 and lactalbumin

Peptide pRL1a and pRL1b binding to hsp70 was evaluated by their competition of complex formation between hsp70 and the unfolded form of lactalbumin as described previously (20). Four micrograms of hsp70 (final concentration, 2.8 mM) and 11 μg of lactalbumin (final concentration, 40 mM) suspended in PBS, pH 7.2 were incubated at 37°C for 30 min with or without gradient doses of pRL1a and PRL1b as indicated in Fig. 3, then resolved by 6% native PAGE and visualized by staining with Coomassie Brilliant Blue R-250. The bands of the stained hsp70-lactalbumin complex were quantitated by densitometry.

Results

Dissociation of the endogenous peptides from gp96, hsp90, and hsp70 purified from RL♂1 and fractionation of the peptides by reverse phase HPLC

The CTL epitope of the RL♂1 mouse leukemia has been identified previously as the octamer epitope (pRL1a), derived from a mutated *akt* gene product (13). A putative precursor of pRL1a, an amino terminal-extended 10 mer peptide (pRL1b), was also identified in this previous study. Association of pRL1a, pRL1b, and other possible precursors with the chaperones hsp70, hsp90, and gp96 was investigated. Fig. 1*A* shows apparently homogeneous preparations of gp96 (*lane a*), hsp90 (*lane b*), and hsp70 (*lane c*) purified from RL♂1 cells and examined by SDS-PAGE and silver staining. In our preparation, another member of the hsp70 family, grp78 was not detected. Immunoblotting of preparations by anti-grp94, anti-hsp90, and anti-hsp70 mAbs was used to confirm their identity and the lack of cross-contamination of the preparations (Fig. 1*B*). Peptides associated with three hsps were isolated as described in *Materials and Methods* and were separated in a C_{18} reverse phase column. The chromatograms of peptides dissociated from gp96, hsp90, and hsp70 are shown in Fig. 2, *a–c*. A distinct profile was observed for the peptides eluted from each hsps preparation. The synthetic 8 mer peptide pRL1a and its putative 10 mer precursor pRL1b were also analyzed, and the chromatograms were shown to elute in fractions 39 and 46, respectively (Fig. 2, *d* and *e*).

Sensitization of P815 cells with endogenous peptides dissociated from hsp70, hsp90, gp96, and L^d molecules, for lysis by CTL clone

All peptide fractions from each of the hsps, L^d molecules, and synthetic peptides pRL1a and pRL1b were used to pulse P815 cells and were tested for recognition by CTL clone Y-15, which recognizes the 8 mer peptide, pRL1a, in association with L^d molecules. We gave careful attention to avoid the carried over peptides with each other; thus, a blank run before running the biological samples was performed in each experiment and confirmed there were no carried over peptides in each preparation. Among RL♂1-derived, hsp70-associated peptides eluted by treatment with ATP (Fig. 3*A-a*) or TFA (Fig. 3*A-b*), significant sensitization activity was observed in fraction 39, which corresponds to the precisely eluted position of L^d-associated or the synthetic 8 mer pRL1a (Fig. 3, *A-e* and *A-f*). A molecule with a molecular mass (*m/z*) of 809, which is precisely the mass of pRL1a, was identified in fraction 39 of the hsp70-associated peptides (Fig. 3, *B-I* and *B-II*). As shown in Fig. 3*A-c*, hsp90 was found associated with three different peptides eluted in fractions 32, 39, and 46, which were able to sensitize P815 cells for lysis by the CTL clone Y-15. The position of fraction 39 has been shown to be identical with that of pRL1a, while fraction 46 contains the synthetic putative precursor 10 mer pRL1b (Fig. 3*A-g*). A molecule with an *m/z* value of 1008, precisely equal to that of pRL1b, was identified in fraction 46 of the hsp90-associated peptides from RL♂1 (Fig. 3, *B-III* and *B-IV*). Thus, hsp90 was associated with the final sized CTL epitope as well as with two other putative precursor peptides, one of which is pRL1b and the other of which was unknown. The size of the precursor peptide eluted in fraction 32 was deduced to be larger than a 10 mer on the basis of the following observations. Peptides shorter than 8 mer pRL1a had no sensitizing activity (data not shown), while the 8 mer pRL1a and 10 mer pRL1b eluted in fractions 39 and 46, respectively. The 9 mer intermediate between pRL1a and pRL1b was observed to elute in fraction 36 or 37 (data not shown). Thus, the sensitizing activity in fraction 32 was >10 mer. The chaperone

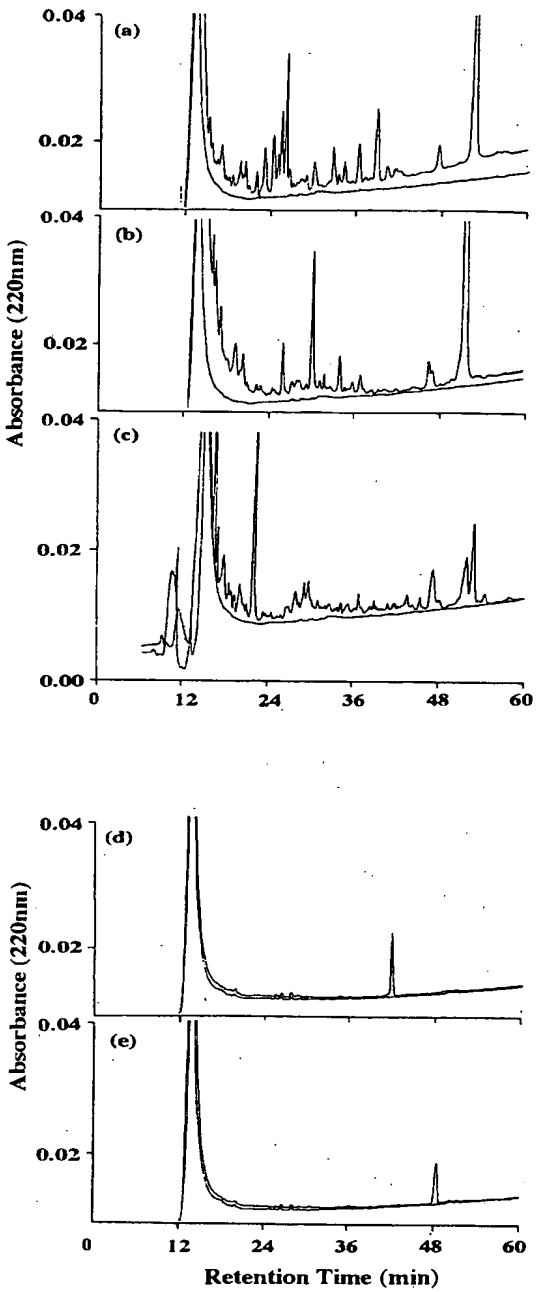

FIGURE 2. The endogenous peptides eluted from gp96 (*a*) and hsp90 (*b*) with 0.2% TFA solution or the peptides eluted from hsp70 (*c*) with ATP treatment and synthetic pRL1a (*d*) and pRL1b peptide (*e*) were analyzed by linear gradient of acetonitrile with a C_{18} reverse phase HPLC column and fractionated at 0.5 ml vol/min. The baseline of each chromatogram is also shown.

gp96 was found associated with both pRL1a and pRL1b eluted in fractions 39 and 46, respectively (Fig. 3*A-d*). No sensitizing activity was detected in fraction 32 or any other fraction among the peptides eluted from gp96. No positive fractions were identified in any peptide fractions eluted from hsp70, hsp90, and gp96 isolated from altered *akt*-negative BALB/c fibrosarcoma Meth A (Fig. 3*A-h*).

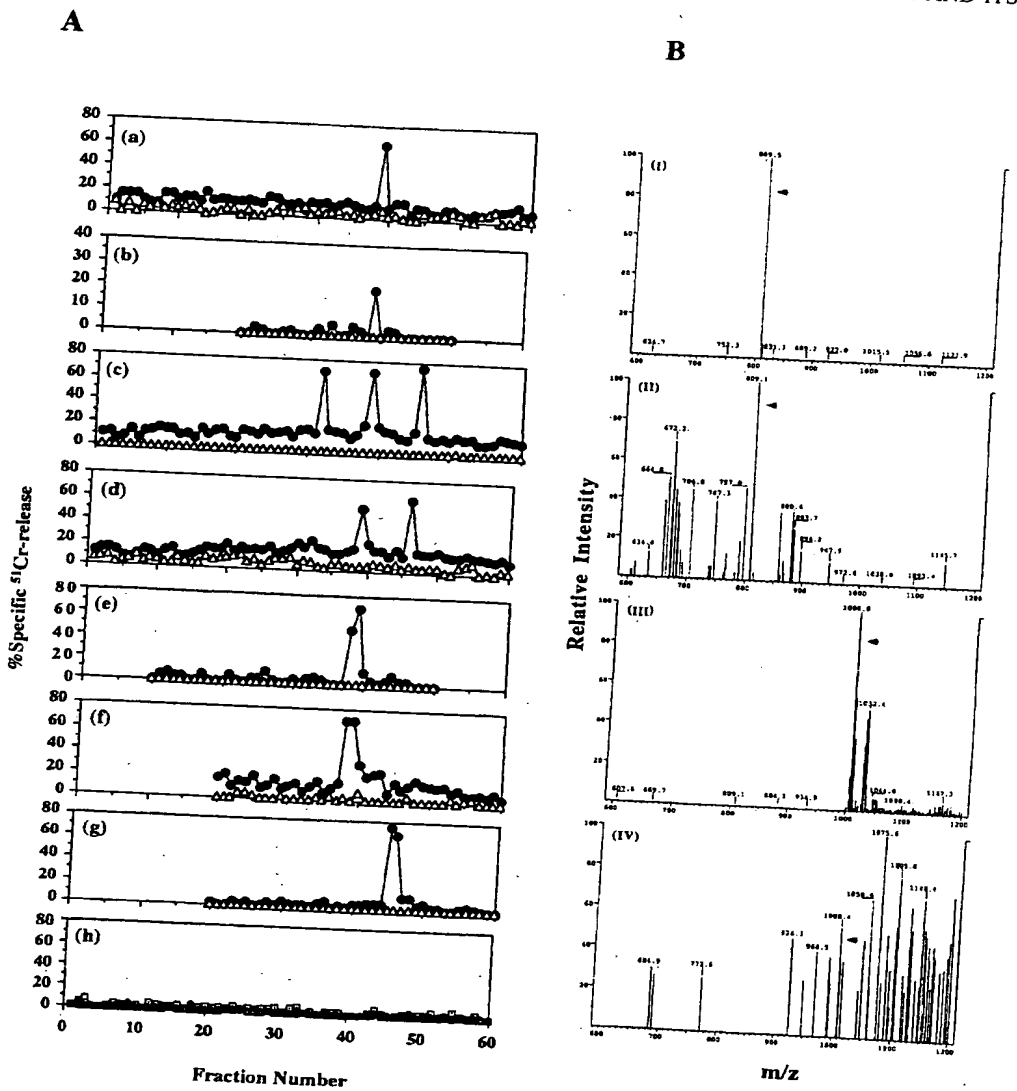

FIGURE 3. Sensitization of P815 cells for lysis by the CTL clone Y-15 by endogenous peptides eluted from hsp70, hsp90, and gp96 purified from RLδ1 cells or Meth A (*A*) and the identification of final sized octamer peptide as well as the putative 10 mer precursor peptide by mass spectrometry analysis (*B*). *A*, The endogenous peptides eluted from hsp70 preparation with ATP (*a*) and TFA (*b*), or the TFA-eluted peptides from hsp90 (*c*), gp96 (*d*), or L^d (*e*) of RLδ1 were fractionated on a C_{18} reverse phase HPLC column as described in Fig. 1, and the fractions were subjected to the ^{51}Cr release assay using P815 cells as target cells and with (●) or without (△) RLδ1-specific CTL clone Y-15 as effector cells. The synthetic peptide pRL1a (*f*) and pRL1b (*g*) were fractionated in the same manner, and each fraction was used for sensitization of P815 cells. Peptides eluted from hsp70 (□), hsp90 (◆), and gp96 (■) isolated from Meth A (*h*) did not show any sensitization of P815 cells. *B*, The synthetic peptide pRL1a (I) and pRL1b (III) or the peptides (II) dissociated from hsp70 in fraction 39 observed in Fig. 2*A-a*, and the peptides (IV) dissociated from hsp90 in fraction 46 observed in Fig. 2*A-c*, were further analyzed by a tandem quadrupole mass spectrometer, and their molecular masses as the *m/z* peaks of single charged ion were identified. The arrowheads (◀) in *I* and *II* indicate a peptide of 809 Da, and the arrowheads in *III* and *IV* indicate a peptide of 1008 Da.

Requirement of serum proteases for hsp90- and gp96-associated precursor peptides to sensitize P815 cells for lysis by the CTL clone

The pRL1a, but not the pRL1b, was detected in TFA-treated immunoaffinity-purified L^d preparations (Fig. 3*A-e*) (13), indicating that this putative precursor peptide pRL1b does not bind L^d. It was presumed that the 10-mer pRL1b and the longer precursor in hsp90-eluted fraction 32 are able to sensitize P815 cells for lysis by the CTL clone Y-15 because of an exopeptidase activity in the serum-containing medium in which the assay was performed. In fact, the synthetic peptide pRL1b was shown to lose its sensitizing activity of P815 cells for lysis by CTLs in the serum-free plain RPMI medium (18). This premise, especially in the case of natural peptides associated with the hsps, was tested directly by carrying out the assay in medium with or without serum. It was observed that (Table I) synthetic peptide pRL1b and fractions 32 and 46 from the hsp90 preparation or fraction 46 from the gp96 preparation could not sensitize P815 cells for recognition by CTL clone Y-15 in the AIM-V serum-free medium. However, the sensitization activities of synthetic pRL1a peptide and peptides in fraction 39 derived from hsp70, hsp90, and gp96 were not impaired in this medium, indicating that the putative precursor peptides are real

Table I. *Hsp90 and gp96 are associated with precursor peptides, whose ability to sensitize p815 cells for lysis by CTL clone Y-15 requires their trimming by serum proteases*[a]

		Pulsed with							
		Synthetic peptides (% cytotoxicity)		Hsp-associated natural peptides (% cytotoxicity)					
					Hsp90			Gp96	
Target Cells	No Peptide	pRL1a	pRL1b	Hsp 70, fraction 39	Fraction 32	Fraction 39	Fraction 46	Fraction 39	Fraction 46
P815 in medium with fetal calf serum	4.9	66.9	73.8	69.0	32.4	66.1	67.1	65.1	28.6
P815 in AIM-V, serum-free medium	11.4	70.5	23.9	74.2	10.4	52.4	7.1	57.2	10.2
(% inhibition)	(0)	(0)	(67.6)	(0)	(67.9)	(20.7)	(89.4)	(12.1)	(64.3)

[a] Sensitization of P815 cells for lysis by Y-15 with the synthetic peptide pRL1a, pRL1b, and hsps-associated natural peptides (fraction 39 eluted from hsp 70; fractions 32, 39, and 46 from hsp90; fractions 39 and 46 from gp96). ^{51}Cr-release assay was performed in the presence or absence of FCS. In the absence of FCS, AIM-V serum free medium (Gibco BRL) was used. The significant difference in sensitizing activity of pRL1b between assays containing the FCS and AIM-V media was observed at the concentrations from 2.5×10^{-7} M to 3.0×10^{-8} M. No significant difference with the pRL1a was observed at the final peptide concentrations from 4.7×10^{-6} M to 2.3×10^{-9} M. The table shows the results at the concentration of 6.2×10^{-8} M of pRL1a and pRL1b. E:T ratio was 20:1.

precursors and must be trimmed at the N-terminus to bind L^d molecules in the medium containing FCS. We cannot determine whether C-terminal trimming of the peptide in fraction 32 from the hsp90 preparation is also necessary.

Binding of pRL1a peptide to hsp70 does not occur after cell lysis

The 8-mer pRL1a was added to a suspension of Meth A cells, which do not express this Ag, and hsp70 preparations were derived from the lysate of such Meth A cells. The concentration of peptide added to Meth A (320 pM final concentration) was severalfold higher than an abundant estimate of the natural peptide in RL♂1. If the association of pRL1a peptide with hsp70 can occur after cell lysis, hsp70 preparations derived from the Meth A cell lysate, to which large quantities of pRL1a have been added, should be found associated with pRL1a. Peptides eluted from such an hsp70 preparation were used to pulse P815 cells, which were then tested for recognition of pRL1a by CTL clone Y-15. No recognition was observed (Table II). In contrast, peptides eluted from hsp70 preparations derived from the RL♂1 could sensitize P815 cells for lysis by CTL clone Y-15 successfully (Table II). Similar studies with hsp90 and gp96 are unnecessary, as association of these hsps with peptides in vitro requires harsh conditions, such as high temperatures or the use of denaturants (17), which were not used in their purification here (see *Materials and Methods*).

Table II. *Binding of peptides to hsp70 does not occur after cell lysis*

P815 Cells Pulsed With[a]	Recognition by CTL Clone Y-15 (% specific ^{51}Cr-release of E:T at 20:1)
No peptides	5.0
pRL 1a synthetic peptide	70.6
hsp70 (RL♂1)-dissociated peptides, fractions 38 + 39	71.2
hsp70 (Meth A)-dissociated peptides fractions 38 + 39	8.4
hsp70 (Meth A) plus exogenous pRL1a -dissociated peptides, fractions 38 + 39	2.7

[a] Peptides dissociated from hsp70 derived from RL♂1 cells, Meth A cells, or Meth A cells plus exogenously added pRL1a synthetic peptide (320 pM in final), were subjected to C_{18} reversed-phase chromatography and fractionated as described in legend to Fig. 1*B*.

The specificity of the observed association of pRL1a to the hsp70 was tested in another manner. Although hsp70 was found in association with pRL1a alone (Fig. 3, *A-a* and *A-b*), studies in vitro indicate that hsp70 can bind equally effectively with both pRL1a and pRL1b; this was demonstrated by competition studies in which the abilities of the two peptides to inhibit formation of the hsp70-lactalbumin complex was tested quantitatively as described in *Materials and Methods* (20). It was observed that generation of the hsp70-lactalbumin complex, as judged by native gel electrophoresis, was inhibited equally in a dose-dependent manner by pRL1a and pRL1b (Fig. 4). Thus, the observed association of pRL1a, but not pRL1b, with hsp70 is significant and occurs in vivo; if the observation were the result of artifactual association of peptides with hsp70 after cell lysis, pRL1a and pRL1b would both be found in association with hsp70 molecules. This is not the case. Thus, pRL1b is not available to hsp70 in vivo.

Discussion

Our studies show that a tumor-specific CTL epitope and its precursors are associated with cytosolic hsp90 and hsp70 and with ER luminal hsps gp96. Hsp90 was found associated with the final sized 8 mer epitope as well as two other precursor peptides, while hsp70 was associated with only the 8 mer epitope, and gp96 was associated with the 8 mer epitope and the 10 mer precursor peptide. The association of hsps with peptides, as reported here, occurs in vivo and does not reflect a random association of peptides with hsps after cell lysis. Thus, exogenously added pRL1a did not associate with endogenous hsp70 (Table II), and pRL1b, although capable of binding hsp70 in vitro, was not found in association with it (Fig. 4). In other studies, it has been demonstrated that addition of a broad array of labeled peptides of diverse sizes and sequences to cell suspensions before lysis does not lead to their association with gp96 (A. Menoret and P. K. Srivastava, manuscript in preparation). Further, as observed here, the patterns of association of peptides were distinct and specific for each hsps; while hsp70 was found associated solely with pRL1a, gp96 is observed associated with both pRL1a and pRL1b, and hsp90 associated with pRL1a, pRL1b, and the larger precursors. In three complete sets of experiments repeated, no deviation from this pattern was detected.

Another issue that needs to be addressed has to do with the possibility that the exact octamer or the putative precursor peptides found associated with the three hsps could have dissociated from

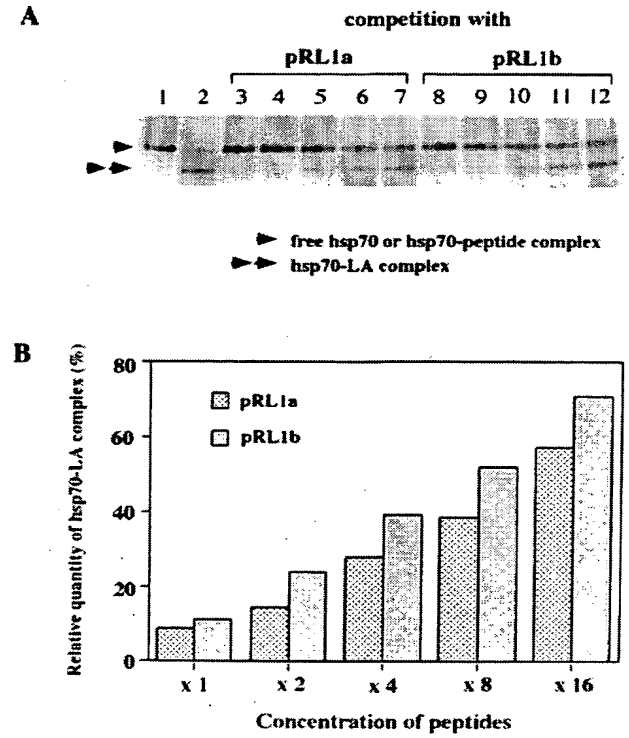

FIGURE 4. Native PAGE analysis of hsp70-lactalbumin (LA) complex formation with or without competition from increased quantities of pRL1a and pRL1b. *A*, The positions of free hsp70, hsp70-peptide complex, and hsp70-LA complex are indicated by arrowheads. *Lane 1*, hsp70 only; *lane 2*, hsp70 and LA (hsp70-LA complex) without peptides; *lanes 3–12*, inhibition of hsp70-LA complex formation by pRL1a (*lanes 3–7*) and pRL1b (*lanes 8–12*) was observed in a dose-dependent manner. Peptide concentrations in individual lanes are: *lanes 3* and *8*, 15×10^{-4} M; *lanes 4* and *9*, 7.5×10^{-4} M; *lanes 5* and *10*, 3.7×10^{-4} M; *lanes 6* and *11*, 1.8×10^{-4} M; *lanes 7* and *12*, 0.9×10^{-4} M. *B*, The relative quantity of hsp70-LA complex (*lanes 3–12*) to that in *lane 2* is calculated by densitometry analysis. The concentrations of peptides are described as the number of twofold dilution starting from 15×10^{-4} M.

the MHC I molecules and associated with the hsps. This possibility is ruled out by a number of considerations. Firstly, the 10 mer pRL1b peptide has been shown unable to bind the L^d molecule (Fig. 3*A-e*) (13), and thus, it cannot bind hsp90 or gp96 after dissociation from L^d molecules. Secondly, the finding that each hsps has a distinct spectrum of peptides associated with it argues forcefully against the possibility of random association regardless of source. Thirdly, the hsps preparations were derived from aqueous (i.e., nondetergent-containing) lysates, where they were purified away from the MHC molecules at the very first step of cell lysis. In addition, the results of other studies, which show that the exact MHC I-binding peptides may be isolated from gp96 preparations derived from cells that do not express the cognate MHC I molecules (11), provide structural evidence that the hsps-associated peptides are not derived from the MHC I-associated peptides. Finally, the observations by Arnold et al. (10) and Suto and Srivastava (8) that hsps preparations can cross-prime against minor histocompatibility Ags and VSV-derived epitopes across the H-2 provide functional evidence that association of peptides with hsps does not derive from or depend upon their association with MHC I molecules.

The association of hsps with the exact-sized MHC I ligand and its precursors might be implicated as the roles of the hsps in MHC I Ag processing/presentation. Peptides associated with the MHC I molecules are eight or nine amino acids long (21), and peptides with different motifs bind specific MHC I alleles (22). The peptides or their precursors originate in the cytosol (23–25) and are transported into the ER, where the association with MHC I molecules occurs (26). The events between the generation of peptides in the cytosol and their final association with MHC I molecules are not fully understood. The peptides are clearly not present in the cytosol in a freely diffusible manner; it has been proposed that molecular chaperones of the hsps family, such as hsp90 and hsp70 in the cytosol and gp96 in the lumen of the ER, constitute a molecular relay line that chaperones the peptides from their generation in the cytosol to their binding to MHC I in the ER (27). Very recently, Shimbara et al. have shown that pRL1b precursor peptide, but not pRL1a, was produced from synthetic peptide by proteasome in the presence of the IFN-γ-inducible activator PA28 (28). To date, pRL1b is the true precursor peptide and needs to be trimmed to become L^d ligand-pRL1a by aminopeptidases. In this context, our data may suggest that the precursor peptide pRL1b (and presumably larger precursors, such as those in fraction 32 of hsp90-associated peptides) are received by hsp90 first, followed by N-terminal trimming of some of the precursor molecules through as yet uncharacterized mechanisms in the cytosol. This idea is consistent with recent reports that demonstrate physical association of hsp90 molecules with the proteasomes (29, 30). However, not all the precursor molecules delivered to hsp90 from the proteasome are completely processed, as the ER luminal hsps gp96 is still observed associated with the pRL1b precursor. In addition, a proportion of precursor peptides may bypass hsp90 altogether and thus go on to associate with gp96. Interestingly, hsp70 molecules are not observed associated with precursor peptides. In light of the reported proteolytic activity of hsp70 (31), this observation might suggest that any precursors that come to associate with hsp70 are rapidly cleaved to the final product. If the transport of pRL1a, pRL1b, and other precursor peptides into the ER is TAP dependent, pRL1b would be expected to be transported at a higher efficiency than pRL1a because of the inhibitory presence of proline at position 2 (32) in the octamer but not in the 10-mer pRL1b. Nevertheless, it is conceivable that the constraints on the ability of TAP molecules to transport peptides do not reflect the situation in vivo, as they have been deduced by monitoring the transport of exogenously added free peptides to TAP-containing vesicles or permeabilized cells. Thus, while free peptides with proline at position 2 may not be transported by TAP molecules, such peptides chaperoned by hsps may be transported by the same TAP molecules more efficiently. The recent observations of Lammert et al. (19, 33), Spee and Neefjes (34), and Marusina et al. (35) are in accord with our present observation and previous speculation that a relay line of hsps chaperones peptides from the point of their generation in the cytosol to their association with MHC I-β_2m molecules in the ER. These authors have demonstrated that peptides associate with a number of peptide-binding proteins in the cytosol (gp100) and in the lumen of the ER (gp96, protein disulfide isomerase, gp120, and gp170). Our studies suggest that hsps are in a position to play an important role in the transport of antigenic peptides to MHC I molecules.

Regardless of the precise mechanisms by which the precursor peptides are transported into the ER, our results are the first evidence showing that hsps localized in distinct intracellular compartments are associated with different sets of precursors of MHC I-binding tumor Ag peptide.

Acknowledgments

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Vaccination of Metastatic Melanoma Patients With Autologous Tumor-Derived Heat Shock Protein gp96-Peptide Complexes: Clinical and Immunologic Findings

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Purpose: **To determine the immunogenicity and antitumor activity of a vaccine consisting of autologous, tumor-derived heat shock protein gp96-peptide complexes (HSPPC-96, Oncophage; Antigenics, Inc, Woburn, MA) in metastatic (American Joint Committee on Cancer stage IV) melanoma patients.**

Patients and Methods: **Sixty-four patients had surgical resection of metastatic tissue required for vaccine production, 42 patients were able to receive the vaccine, and 39 were assessable after one cycle of vaccination (four weekly injections). In 21 patients, a second cycle (four biweekly injections) was given because no progression occurred. Antigen-specific antimelanoma T-cell response was assessed by enzyme-linked immunospot (ELISPOT) assay on peripheral blood mononuclear cells (PBMCs) obtained before and after vaccination. Immunohistochemical analyses of tumor tissues were also performed.**

Results: **No treatment-related toxicity was observed. Of 28 patients with measurable disease, two had a complete response (CR) and three had stable disease (SD) at the end of follow-up. Duration of CR was 559+ and 703+ days, whereas SD lasted for 153, 191, and 272 days, respectively. ELISPOT assay with PBMCs of 23 subjects showed a significantly increased number of postvaccination melanoma-specific T-cell spots in 11 patients, with clinical responders displaying a high frequency of increased T-cell activity. Immunohistochemical staining of melanoma tissues from which vaccine was produced revealed high expression of both HLA class I and melanoma antigens in seven of eight clinical responders (two with CR, three with SD, and the three with long-term disease-free survival) and in four of 12 nonresponders.**

Conclusion: **Vaccination of metastatic melanoma patients with autologous HSPPC-96 is feasible and devoid of significant toxicity. This vaccine induced clinical and tumor-specific T-cell responses in a significant minority of patients.**

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NO EFFECTIVE THERAPY for metastatic melanoma is currently available. Several phase II studies of biochemotherapy have reported 40% to 50% clinical response rates.[1] Recently, one[2] but not another[3] phase III trial confirmed a significantly higher response rate of biochemotherapy as compared with chemotherapy alone.[2] However, these treatments were associated with high toxicity and did not result in concurrent improvement in overall survival.[2,3]

Several vaccine approaches have been tested. Most recently, patients have been vaccinated with peptides (with or without adjuvants, or pulsed on autologous dendritic cells) derived from proteins expressed by melanoma cells. The clinical response rate in these studies has ranged between 5% and 30%.[4-6] In one trial where patients received concurrent administration of high-dose interleukin-2,[7] a response rate of 42% was achieved. In virtually all of these

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studies, complete responses are rare and, with an exception,[5] of short duration, frequently less than 6 months. As another approach, the use of gene-modified autologous or allogeneic melanoma cell vaccines has resulted in response rates of 10% to 20%.[8] In many trials, patients have been monitored for tumor-specific T-cell responses to vaccination by a variety of assays. Such immunologic responses have been usually detected in a low percentage of patients, and when they have been detected, no correlation between immunologic responses and clinical outcomes has been observed.[5,7,9]

In this study, we have immunized melanoma patients with vaccines consisting of heat shock protein-peptide complexes. Heat shock proteins (HSPs) are the most common and abundant proteins in all forms of life. They come in different families characterized by members of similar molecular mass (such as hsp70 and hsp90). Previous work has demonstrated that purified, apparently homogeneous preparations of gp96, hsp70, hsp90, and certain other HSPs are actually noncovalent complexes of HSPs and peptides (see Srivastava[10]). The peptides are derived from the proteins expressed in the cells from which the HSPs are purified and include normal self-peptides and antigenic peptides. This phenomenon has been demonstrated in mouse[11,12] and human tumors.[13] In the latter, hsp70-peptide complexes extracted from melanoma cells have been found to contain well-known peptides on the basis of their ability to stimulate antigen-specific $CD8^+$ T cells from melanoma patients' peripheral blood mononuclear cells (PBMCs).[13] Vaccination with autologous tumor-derived HSP-peptide complexes has been shown to result in both prophylactic and therapeutic antitumor activity in multiple animal tumor models in multiple genera.[14,15] Furthermore, a pilot clinical study of vaccination with HSP peptide complex-96 (HSPPC-96) isolated from autologous nonmelanoma tumors suggests that a specific antitumor immunity can be generated, even in advanced cancer patients.[16]

The immunogenicity of tumor-derived HSP-peptide complexes, like the immunogenicity of experimentally induced tumors of mice and rats, has been shown to be individually tumor specific and not tumor type specific. These observations have led to the conclusion that the relevant tumor-antigenic, immunoprotective peptides are derived from unique rather than shared tumor antigens.[17] This is the basis of the vaccination protocol in this study, where each patient is vaccinated with HSP-peptide complexes isolated from his or her own tumor. In the present study, we have evaluated whether vaccination with autologous, tumor-derived HSPPC-96 (Oncophage; Antigenics, Inc, Woburn, MA) is feasible and safe, and whether such vaccination elicits immunologic and/or clinical response in the autologous host.

PATIENTS AND METHODS

Patients and Treatment

Between February 1999 and January 2000, 64 stage IV (American Joint Committee on Cancer) metastatic melanoma patients were enrolled on the study and had surgical resection of tumor tissue for vaccine production. Patients were enrolled at four Italian centers: the Istituto Nazionale Tumori (n = 32) and the European Institute of Oncology of Milan (n = 21), the Regional Center of Oncology of Aviano (n = 7), and the Scientific Institute of Tumors of Genoa (n = 6). Of these patients, 39 received at least one cycle of vaccination, and were assessable. Eligibility criteria for vaccination included the following: (1) histologically verified melanoma with resectability of at least one lesion that was able to provide the necessary amount of nonnecrotic neoplastic tissue (3 g for vaccine preparation and 1 g for immunologic assays); (2) a performance status (Zubrod) of 2 or less; (3) life expectancy of at least 20 weeks; (4) normal WBC and platelet counts, and hemoglobin level greater than 10 g/L; (5) bilirubin less than 1.5 times normal, ALT less than four times normal, and adequate renal function with serum creatinine of less than two times normal; (6) fully recovered from prior anticancer therapy with at least a 4-week interval from the last administration of prior anticancer treatment; and (7) a positive response to recall antigens (Multitest Mérieux; Imtix, Milan, Italy) as a sign of sufficient immune function.

All patients underwent clinical and radiographic staging before treatment. Patients were excluded from the study if they had active brain metastases, had concomitant autoimmune or malignant diseases or were receiving concurrent anticancer therapy, required steroid drugs, or had a history of serious intercurrent medical illnesses. Women of childbearing potential required a negative serum pregnancy test before entry onto the study and agreed to use an effective method of contraception while on treatment. All patients gave written informed consent to participate in the study.

The clinical protocol was approved by the Internal Scientific Review Board and by the Independent Ethics Committee of the Istituto Nazionale Tumori of Milan and of the other participating centers. Patients were vaccinated with autologous HSPPC-96 starting 5 to 8 weeks after resection of tumor metastases with 5 or 50 μg of vaccine given at weekly intervals either intradermally or subcutaneously. Patients were randomly assigned to either dose or route of administration of the vaccine. After four vaccinations (first cycle), and if no progression occurred, patients could receive four additional injections once every 2 weeks (second cycle) if vaccine was available. This occurred in 21 patients; in addition, four patients (02-007, 02-021, 03-003, and 03-005) underwent two to three additional monthly immunizations of HSPPC-96 as a third cycle of vaccination.

Patients were monitored for toxicity, including complete clinical evaluation, standard blood tests including differential blood counts, serum chemistry, urinalysis, cardiac, liver and renal functions, and autoimmune reactions (antimicrosomal and antithyroglobulin antibodies, T_3, free T_4, rheumatic factor, and antinucleoprotein and antimitochondrial antibodies). Ophthalmologic examinations were carried out at 4, 8, and 12 weeks to assess possible autoimmune reactions caused by melanoma/retina cross-reacting differentiation antigens.

Tumor evaluation was performed before resection of metastatic melanoma, at the pretreatment visit (baseline evaluation or visit 1 [V1]), at weeks 4 and 8 of the study, and thereafter every 3 months and as indicated. This was by the same sequential diagnostic imaging method. The size of tumor lesions was evaluated as the product of the largest perpendicular diameters of the lesion and recorded. Clinical

responses were defined as less than progressive disease, which spans the spectrum from stable disease (SD) to complete response (CR). Disease-free survival (DFS) was measured from the day of onset of vaccination until relapse in patients that were rendered disease-free by surgery.

Preparation of Vaccine

Autologous HSPPC-96 vaccine was prepared from tumor samples of each patient as reported for mouse tumors.[18] Briefly, macroscopically nonnecrotic tumor tissue was obtained under sterile conditions from the operating room, weighed, and immediately frozen in liquid nitrogen. It was shipped to Antigenics and processed under good manufacturing practice conditions. Preparations were considered to be of acceptable quality only if all of the following three conditions were met: (1) the major protein band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis was 96 kDa; (2) this band could be immunoblotted with an anti-gp96 monoclonal antibody (mAb); and (3) the preparations were sterile and their endotoxin levels were within the levels approved by the Food and Drug Administration. All the preparations used to vaccinate patients met these criteria.

Evaluation of Immune Response

HLA typing. Serologic typing for HLA-A, HLA-B, and HLA-C was performed by the standard two-stage complement-dependent microcytotoxicity test. For molecular typing, genomic DNA was purified from protease-treated PBMCs with QIAamp DNA kit (Qiagen, Germany), and HLA typing was performed by amplification with sequence-specific primers (Dynal, Bromborough, United Kingdom).

Skin response (delayed-type hypersensitivity). Skin reaction at the site of vaccination was assessed 1 hour after every injection by the attending physician and after 24 and 48 hours by the patient on instruction. Delayed-type hypersensitivity was carried out by subcutaneous injection of 3×10^4 or 10×10^5 autologous tumor cells (when available) derived from melanoma lesions by mechanical processing. Tumor cells were then irradiated (150 Gy) and tested for sterility by routine microbiologic assays. Cell suspensions were aliquoted, stored in liquid nitrogen, and thawed before injection. Autologous irradiated (50 Gy) and autologous PBMCs, obtained from the blood by Ficoll (Pharmacia Biosystems, Uppsala, Sweden) gradient, were also injected as negative control immediately before the first, fourth, and fifth vaccinations.

Eighty to 100 mL of heparinized blood for immunologic assays was obtained by preparation of a buffy coat from each patient before vaccination (V1) and at V5 (fourth vaccination) and V6 (4 weeks afterward). In patients who received additional vaccinations, blood was obtained also at V7, V9, V10, or V11. The PBMCs were isolated by Ficoll gradient centrifugation and frozen in aliquots in liquid nitrogen. The following tests were performed at the Istituto Nazionale Tumori for all patients to assess their melanoma-specific immune response. PBMCs of only 23 subjects could be analyzed in part for lack of availability of PBMCs, and in part for the lack of appropriate melanoma cell target because of the rare HLA allele of patients.

Enzyme-Linked Immunospot Assay

The enzyme-linked immunospot (ELISPOT) assay allows the direct testing of antigen recognition by patients' T cells and has been used in melanoma patients vaccinated with different peptides.[19-21] Blocking experiments were performed by preincubating target cells for 30 minutes at 37°C with the anti–class I HLA antibody W6.32 or the anti–class II HLA antibody L243. To see whether HSPPC-96 could stimulate PBMCs, autologous monocytes were separated from PBMCs by overnight adherence in 10% fetal calf serum (FCS) RPMI 1640 at 37°C, recovered by mechanical removal, and incubated for 2 hours at 37°C with 2 μg/mL autologous tumor-derived HSPPC-96 (from the same preparations used for the vaccine). Monocytes were then directly used as target in ELISPOT assay with or without previous incubation with W6.32 or L243 mAb for 30 minutes on ice. As effectors, nonadherent cells from autologous PBMCs obtained either before (V1) or after (V5) vaccination were used.

To control the ELISPOT assay, the anti-Melan-A/MART-1_{27-35} cytotoxic T-lymphocyte clone A42[22] in the presence or absence of relevant targets (T2 cells pulsed with Melan-A/MART-1_{27-35} peptide and the Melan-A/MART-1_{27-35}^+ melanoma line 501mel) was always included at the concentration of 400 cells/well. The HLA-$A2^+$ lymphoblastoid cell line T2 carries a TAP mutation, which prevents endogenous peptide presentation but allows external peptides to be presented by its class I HLA empty molecules. After an additional incubation for 20 hours at 37°C and 5% CO_2, plates were washed with filtered phosphate-buffered saline (PBS). Wells were then incubated for 2 to 4 hours at room temperature with 50 μL/well of biotinylated mouse antihuman interferon gamma (IFN-γ) mAb (Mabtech, Nacka, Sweden) at the concentration of 1 μg/mL in filtered PBS with 0.5% FCS. Wells were then washed with PBS and 100 μL/well of streptavidin alkaline phosphatase (Mabtech) diluted 1:1,000 in filtered PBS–0.5% FCS was then added. After 1 hour incubation at room temperature, plates were washed extensively and 100 μL/well of substrate (BioRad, Hercules, CA) was added. Color development was stopped by washing in tap water when dark spots emerged (up to 30 minutes). Plates were then left to dry overnight at room temperature and spots were counted by a computer-assisted ELISPOT reader (Bioline, AID, Torino, Italy). For HLA-blocking experiments, PBMCs were thawed 18 hours before their use and depleted from monocytes by overnight adherence in 10% FCS RPMI 1640 at 37°C; nonadherent cells were used as effectors at 2×10^5 cells/well. Autologous or allogeneic HLA-matched melanoma cells or HSPPC-96/pulsed monocytes were preincubated with either W6.32 or L243 antibodies for 30 minutes on ice and used as targets (2×10^4 cells/well).

Indicated spot numbers per seeded PBMCs represent mean values of three or six replicates. To calculate the number of PBMCs responding to the antigen (tumor cells, peptide or HSPPC-96/pulsed monocytes or T2 cells) by IFN-γ release, a background was subtracted. Background was estimated according to the various combination of stimulator and responder. When tumor cells were used for stimulation, background was the number of IFN-γ spots associated with PBMC responders alone. For the ELISPOT assay with HSPPC-96/pulsed monocytes, background subtracted was also the spots of PBMCs alone, whereas the background of monocytes alone is indicated in Fig 2. For statistical evaluation, a Student's t test for unpaired samples was used to compare prevaccine and postvaccine spots of the same patient. Values of $P < .05$ were considered as significant.

Melanoma and Other Cells or Cell Lines

Tumor cell suspensions were obtained by mechanical processing of melanoma lesions. Enzymatic digestion with a pool of DNAse I, collagenase type IV (1 μg/mL) (Biowhittaker Europe, Belgium), hyaluronidase type IV (Sigma) (1 μg/mL), and trypsin-EDTA (Biowhittaker) was performed in some cases to achieve higher cell recovery. Tumor cells were then aliquoted, frozen, and stored in liquid nitrogen. Melanoma cells expressing different HLA alleles and used as targets in the ELISPOT assay were derived from patients accrued onto the study. In addition, the melanoma lines 501mel and 624.38mel were

Table 1. Characteristics of Assessable Metastatic Melanoma Patients

Characteristic	No. of Patients			Total No. of Patients
	With Measurable Disease		Disease-Free	
Total no. of patients	28*		11†	39
Sex				
Male	11		7	18
Female	17		4	21
Performance status (Zubrod)				
0	8		1	9
1	19		10	29
2	1		0	1
Age, years				
Median	55.5	40	51.3	
Range	30-75	29-71	29-75	
Site of metastases				
Single metastasis	11		10	21
Cutaneous and/or subcutaneous	8		1	9
Lymph nodes	0		6	6
Visceral	3		2	5
Parotid	0		1	1
Multiple metastases	17		1	18
Subcutaneous and lymph nodes	3		0	3
Subcutaneous and/or lymph nodes + visceral	14		1	15
Previous treatments				
Surgery only	8		5	13
Chemotherapy only	2		1	3
IFN-α	5		2	7
Chemotherapy + IFN-α ± interleukin-2	12		1	13
Chemotherapy + IFN-α ± radiation therapy	3		0	3

*Of the 28 patients, 13 received subcutaneous and 15 intradermal injections, respectively.
†Of the 11 patients, five received subcutaneous and six intradermal injections, respectively.

used as HLA-A2$^+$ target cells,[22] whereas Me15932 was used as an HLA-A3$^+$ tumor cell line.[23] The colon carcinoma cell line Colo206, expressing both HLA-A2 and HLA-A1 alleles, was a gift from C. Scheibenbogen, MD (B. Franklyn University, Berlin). Tumor cell lines were maintained in RPMI 1640 with 10% FCS unless otherwise indicated.

Immunopathology of the Resected Melanoma Metastases

Routine histologic diagnosis was performed for all tumor samples used for vaccine production. In addition, histologic and immunohistochemical evaluation was performed in 21 of the 39 assessable patients at the time of tumor procurement for vaccine preparation. Classification of the metastatic foci on the basis of the "brisk/nonbrisk" system for tumor-infiltrating lymphocytes[24,25] was performed on routine hematoxylin and eosin–stained slides. In addition, this was correlated with immunohistochemical analysis using the routine immunoperoxidase technique. Tissue sections (1 to 2 μm thick) were stained with the following antibodies: CD3 and CD4 (Novocastra Labs, Newcastle on Tyne, United Kingdom); CD8 (DAKO A/S, Glostrup, Denmark); CD45RO (clone UCHL1, DAKO A/S); CD56 NCAM (Sigma); HMB45 (DAKO A/S); S-100 (DAKO A/S); Melan-A/MART-1 (clone A103, Novocastra Labs); HLA-DR (clone LN3, Biotest AG, Dreieich, Germany); the anti–HLA-A,-B,-C framework mAb HC-10 (kindly provided by Soldano Ferrone, MD, Roswell Park Institute, Buffalo, NY). Tissue sections subjected to the same treatment but without incubation with primary mAb were used as negative controls. Positive controls were a reactive lymph node for the lymphocyte markers and for HLA-DR and HLA-A, HLA-B, and HLA-C. Previously well-documented melanoma cases were used as positive controls for the melanoma antigens. If all tumor-infiltrating lymphocytes (TILs) were of T phenotype, the CD3$^+$ population was considered to be 100%. This was used as a baseline for a semiquantitative count of CD4, CD8, and CD45 cells that were estimated as a percentage of the CD3$^+$ counterparts. HLA-A, HLA-B, and HLA-C and melanoma antigens (gp100 or Melan-A/MART-1) were arbitrarily considered as downregulated when less than 20% of tumor cells were positive and highly expressed when at least 50% of tumor cells were positive after staining with the given antibody.

RESULTS

Patients and Vaccination

Of 64 patients (Table 1 lists demographics and clinical data) who underwent surgery for removal of melanoma lesions, 39 received one (n = 18) or two (n = 21) complete cycles of vaccination. Others (n = 25) did not receive a complete cycle of vaccination for reasons listed in Table 2. Of the 39 patients who completed one or two cycles of vaccination, 26 were heavily pretreated with a variety of

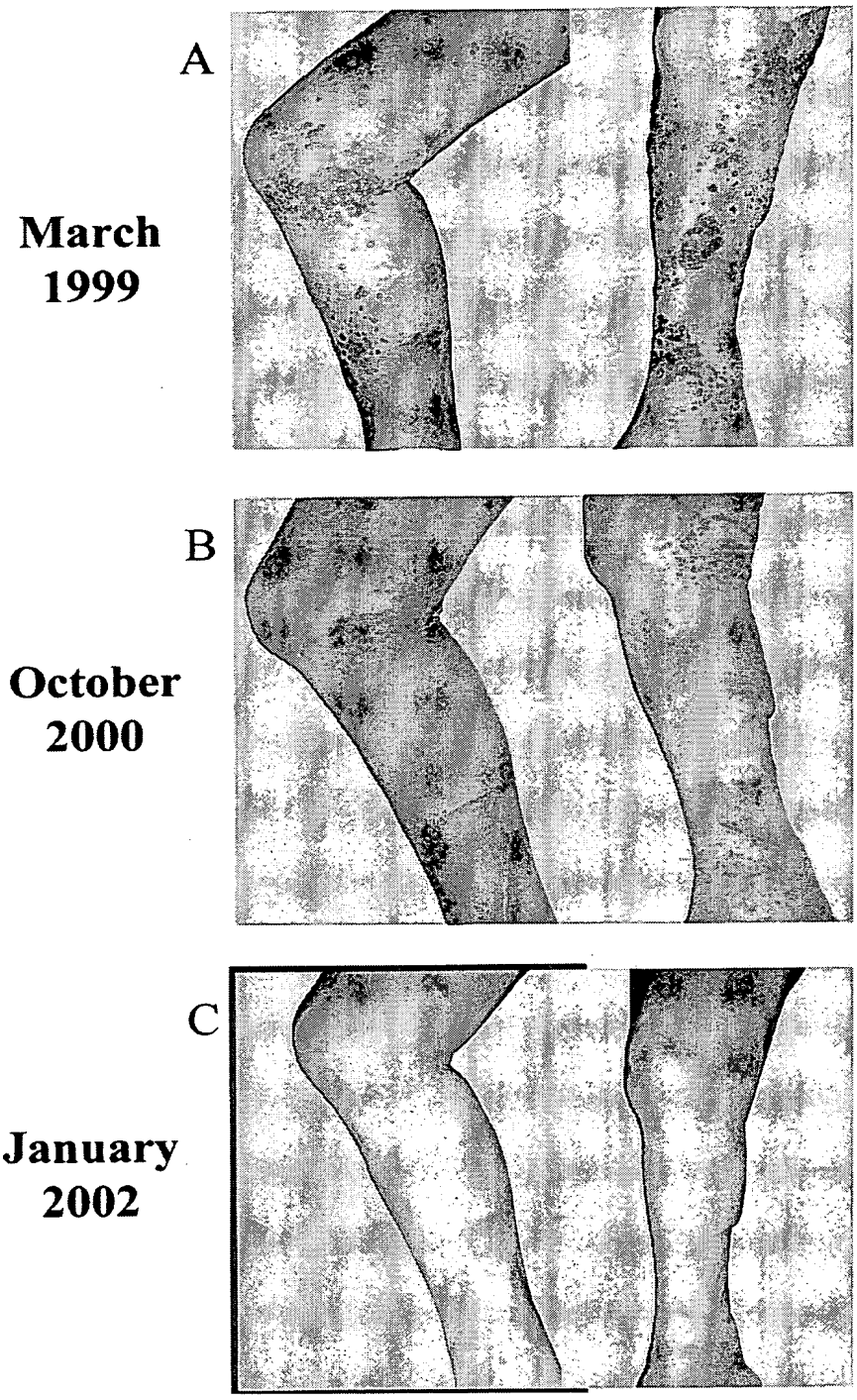

Fig 1. Complete regression of multiple cutaneous metastases in the right limb of patient 01-007 after vaccination. (A) Before vaccination (March 1999). (B) Sixteen months after onset of vaccination (October 2000). (C) Thirty-two months after onset of vaccination (January 2002).

Table 2. Frequency and Causes of Dropout Occurring During Vaccine Production and Before the Conclusion of the First Vaccination Cycle of 64 Eligible Melanoma Patients

Cause	No. of Patients Dropped Out	%
Disease progression	9	14
Death	3	4.7
Chemotherapy	1	1.5
< 3 g of tissue	5	7.8
Insufficient amount of vaccine	7	10.9
Total	25	39

systemic treatments (Table 1). Others (n = 13) underwent surgery alone. Classified by another parameter, 28 patients had residual measurable disease after surgery and 11 were rendered disease-free (Table 1). As mentioned in Patients and Methods, patients were randomized onto 5 or 50 μg HSPPC-96 per vaccination and onto intradermal and subcutaneous vaccination groups.

Toxicity

No toxicity attributable to Oncophage was observed in any of the 39 patients, with the exception of small (less than 5 mm) erythemas and/or induration at vaccination sites, all of which disappeared in less than 24 hours and were attributed to the syringe trauma.

Overall Clinical Response

Of the 28 patients with residual measurable disease, there were two with CR and three with SD. The duration of the CRs (as of April 2002) is 24 and 38+ months, and the duration of the SDs is 153, 191, and 272 days, respectively. The median time to progression and median survival time of the 28 patients was 29 and 402 days, respectively.

Of the 11 patients rendered disease-free by surgery, DFS ranged from 29 to 642 days (median, 117 days). Three patients (01-017, 01-019, and 03-003) remained disease-free for 252, 366, and 642 days, respectively. A clear correlation between dose/route and clinical responses could not be discerned.

CRs

Patient 01-007. This patient (a 75-year-old female) had primary melanoma of the right leg resected in August 1997. In January 1998, in-transit metastases were removed and right radical dissection of inguinal lymph nodes was performed. When new cutaneous lesions appeared, she was given IFN-α and subsequent chemotherapy (cisplatin, vinblastine, and dacarbazine) followed by local radiotherapy. Despite treatment, a gradual progression of the cutaneous disease that enveloped most of the lower extremity and abdominal wall occurred. In March 1999, some of the lesions were resected for vaccine production; vaccination with 5 μg of HSPPC-96 subcutaneously was started in May 1999. At that time, the patient displayed multiple cutaneous metastases, single or confluent, different in size, and some with ulceration and bleeding (Fig 1). Two (confluent nodules) were taken as parameter lesions: lesion 1, on the lower third of the leg (5 × 3 cm), and lesion 2, on the internal side of the right knee (12 × 5 cm) (Fig 1A). During immunization, most of the lesions remained stable; 1 month after the last vaccine, all lesions appeared flattened, lighter, and slightly decreased in size (lesion 1, 4.5 × 2 cm; lesion 2, 10 × 2 cm), and no new nodules appeared. In October 1999, two of the lesions were biopsied and no viable tumor cells were found; there were macrophages containing melanotic pigment, and rare lymphocytes and plasma cells. In addition, no visceral disease was detected by computed tomographic (CT) scan. Since then, a further flattening and decrease in size of the metastases was observed, with no appearance of new lesions in other sites (Fig 1B). At the end of January 2002, the skin of tumor-bearing areas appears normal (Fig 1C). The patient has remained disease-free (April 2002) for a total of 38 months.

Patient 02-003. This patient (a 73-year-old female) had resection of a primary melanoma in 1996 and of locoregional recurrence in January 1999. She was then accrued onto a randomized trial and treated for 1 year with intermediate-dose IFN-α. In May 1999, she developed diffuse subcutaneous, cutaneous, and mucosal metastases. During staging, metastases were detected also in the lung, liver, spleen, and mediastinal and retroperitoneal lymph nodes. None of the metastatic lesions exceeded 2 cm in maximum diameter. In July 1999, surgery was performed to remove some of the larger and more accessible subcutaneous lesions, and vaccine was produced. In September 1999, she began the first cycle of vaccination with 50 μg of HSPPC-96 delivered subcutaneously. At the end of the first cycle (four injections), the patient presented with a clear CT scan of the lung, and all cutaneous and mucosal lesions appeared flattened. The patient completed treatment up to the second cycle (March 2000), as no more vaccine was available; 2 months later, while still in CR, she began a daily treatment with IFN-α at low dose, which was continued up to January 2001, when it was interrupted because of the patient's low compliance. At the end of vaccination, two lesions were biopsied and no residual neoplastic cells were present: only S-100$^+$ and CD68$^+$ pigment-rich macrophages were found. CT scans demonstrated the early reduction in size and then the complete disappearance of all the remaining visceral lesions. The patient remained without evidence of disease until May 2001, when she developed a

brain metastasis, although no other visceral or cutaneous lesions were evident. She then died in August 2001.

Stable Disease

Three patients (01-010, 01-021, and 03-005) had SD lasting 191, 272, and 153 days, respectively.

Re-presentation of gp96-Chaperoned Melanoma Antigens by Monocytes to Patients' Lymphocytes

Previous workers have demonstrated that gp96- or hsp70-peptide complexes are taken up by antigen-presenting cells and the peptides are re-presented by the class I major histocompatibility complex (MHC) of the antigen-presenting cells.[10,12,26,27] These class I MHC–peptide complexes can stimulate cognate $CD8^+$ T cells. This observation, first made in the murine system,[11] was made in the human system with respect to hsp70-chaperoned peptides.[13] We have inquired here whether the human melanoma-derived gp96-peptide complexes are also taken up and re-presented by human monocytes and whether the resulting class I MHC–peptide complexes can stimulate the T cells of the immunized patients. This analysis could be carried out only for two patients because of constraints of availability of HSPPC-96 for testing in vitro. It was observed (Fig 2) that autologous monocytes could re-present gp96-chaperoned peptides to the PBMCs of patients and that PBMCs obtained after vaccination responded (as assessed by ELISPOT assay) more vigorously after vaccination than before vaccination. The stimulation of PBMCs was blocked by the anti–class I HLA (W6.32) but not by an anti–class II HLA (L243) antibody. When colon cancer cell–derived HSPPC-96, instead of the autologous melanoma-derived gp96, was used in the re-presentation assay, no specific recognition was observed, although a background of nonspecific (non-inhibitable) recognition, probably mediated by natural killer (NK) cells, could be detected (Fig 2). The increased reaction observed in PBMCs of patient 01-022 after adding W6.32 could be because of masking of class I HLA that can result in increased NK cell lysis of melanoma cells.[28] This experiment thus indicates that Oncophage preparations do contain antigens that can effectively stimulate the immune system of melanoma patients.

Immunologic Response in HSPPC-96 Vaccinated Patients: Increased Recognition of Autologous or HLA-A–Matched Allogeneic Melanoma Cells by Patients' PBMCs

Patients were monitored to determine whether HSPPC-96 vaccine could increase the frequency of melanoma-specific T cells in peripheral blood. Fresh PBMCs were obtained before and at different times after vaccination, and ELISPOT assay for IFN-γ was performed using melanoma cells

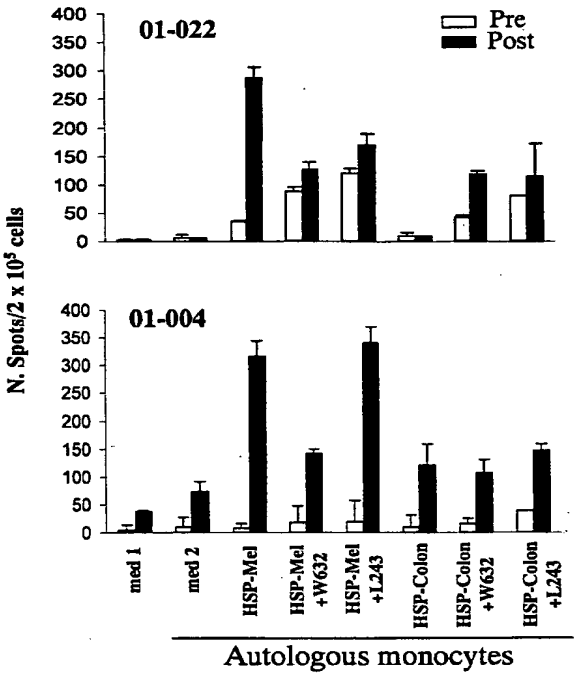

Fig 2. Re-presentation of gp96-chaperoned peptides by autologous monocytes to PBMCs (before and after vaccination) in two patients. Med, medium (negative control); Med 1, effector lymphocytes only; Med 2, autologous monocytes and effector lymphocytes without HSPPC-96.

as targets. Data are reported in Fig 3 as number of positive spots in 1.6×10^5 PBMCs as analyzed in the presence of autologous melanoma cells (either from fresh tumor suspensions or cell lines, when established) or allogeneic HLA-A–matched melanoma lines, when viable tumor cells from autologous lesions were not available. PBMCs from a total of 23 patients were tested at V1 (baseline); at V5 (after the fourth vaccination, first cycle); at V6 (4 weeks later) for patients who received a single cycle of immunization; and in addition at V7, V8, and V10 for patients also receiving the second cycle. Data shown in Fig 3 refer to V1 as prevaccine and mostly to V5 as postvaccine samples. However, in four cases, immunization occurred later during treatment and was detectable at V6 in two subjects and at V7 and V8 in the two other patients, respectively. Despite the known heterogeneity in the baseline antimelanoma reactivity evaluated by ELISPOT,[21] 11 of 23 subjects tested (47.8%) displayed a statistically significant increase ($P < .05$ or $< .01$) in the ability of postvaccination PBMCs to release IFN-γ in response to either autologous or HLA-A–compatible allogeneic melanoma cells, and were considered

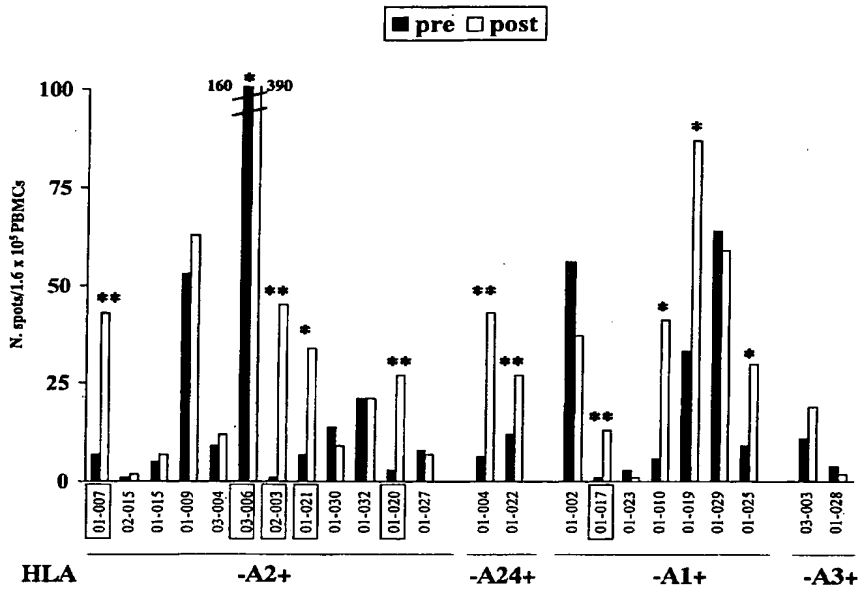

Fig 3. T-cell–mediated antimelanoma activity of PBMCs of 23 metastatic melanoma patients, as evaluated by ELISPOT assay before and after vaccination. The use of HLA-A–compatible allogeneic melanoma cells is indicated by boxed patients' number. Statistical significance was assessed by the Student's *t* test. $^{*}P < .05$, $^{**}P < .01$.

immunologic responders. These patients included four of four (two with CR and two with SD) clinically responding individuals tested (patients 01-007, 02-003, 01-021, and 01-010), and two of three long-term DFS patients (01-017 and 01-019) (Fig 3). The increase of IFN-γ release of these 11 subjects was detected at least at two time points after vaccination and was additionally observed during the second cycle of vaccination in eight of them. Three patients (01-004, 01-019, and 03-006) experienced a transient boost of tumor recognition detectable only at V5. However, even five of the remaining 16 clinically nonresponding patients (03-006, 01-020, 01-004, 01-022, and 01-025) showed an increased antimelanoma T-cell activity (Fig 3). When autologous tumor cells could be used, the observed increase was additionally detected against one or more HLA-A–matched melanomas, suggesting that immunization might have occurred toward shared tumor-associated antigens (data not shown). To evaluate whether such increased reactivity was directed specifically to melanoma cells, IFN-γ release in response to the HLA-$A2^{+}$– and HLA-$A1^{+}$–matched colon carcinoma line Colo206 was analyzed. No increase was evident in the number of postvaccine spots generated by PBMCs as compared to pretreatment samples (data not shown). No differences were found in frequency of immune response in patients who received the vaccine by subcutaneous or intradermal routes or who received 5 *v* 50 μg of HSPPC-96.

As further evidence of antigen-specific T-cell involvement in increased tumor recognition, fresh PBMCs of the 11 immunologically responding patients were tested by ELISPOT with irradiated autologous or HLA-matched melanoma cells in the presence of anti–class I (W6.32) or anti–class II (L243) HLA antibodies. In all cases, T-cell cultures recognized autologous and/or HLA-matched melanoma cells, and such recognition was significantly inhibited with W6.32 but not L243 antibody, indicating an anti–class I HLA-restricted, $CD8^{+}$-mediated, T-cell recognition of melanoma antigens. For the 11 subjects, the inhibition of spot formation ranged from 40% (patient 01-025) to 93% (patient 02-003), with nine of 11 cases in which inhibition was higher than 60%.

It is of note that the frequency of clinically responding subjects, broadly considered to include also those with SD and long-term DFS, displaying a T-cell response was higher than that of the group of patients whose disease progressed (Table 3). No delayed-type hypersensitivity reactions were detected, as also reported by Janetzki et al[16] during vaccination of 12 nonmelanoma patients given autologous HSPPC-96.

Immunopathology of the Resected Melanoma Metastases

Among the 21 samples of assessable patients examined, there was a good correlation between the hematoxylin and eosin evaluation and CD3 staining for TILs. In the cases

Table 3. Antimelanoma T-Cell–Specific Response* and Clinical Response

Group of Patients	Clinical Status	T-Cell Response	
		No. of Tested Patients	No. of Positive Patients
Clinical responders	Measurable disease	4†	4
	Long-term disease-free	3	2
	Total	7	6
Clinical nonresponders	Measurable disease	12	4
	Surgically rendered disease-free	4	1
	Total	16	5‡

*As evaluated by ELISPOT.
†Refers to two CR and two SD patients.
‡$P < .01$ (χ^2 test) compared with clinical responders.

labeled as absent, immunohistochemistry showed either absence of or only rare lymphocytes within the tumor, whereas the brisk cases displayed a diffuse distribution of $CD3^+$ cells (Table 4). In the nonbrisk cases, the TIL population was somewhere between the two ends of the spectrum and were labeled as scattered. In almost all cases (19 of 21), the intratumoral lymphocytes showed a cytotoxic ($CD8^+$) and memory (CD45RO) phenotype. In two cases (01-001 and 01-022), the intratumoral lymphocytes marked only for CD45RO. The remaining two cases (01-027 and 01-006) showed complete absence or few intratumoral lymphocytes. Therefore, a percentage could not be estimated. In the cases where $CD4^+$ cells were seen, they were either at low percentage (20% to 30% of TILs) or the sample was a lymph node, in which case the $CD4^+$ population was interpreted as being part of the "host" lymph node rather than true intratumoral lymphocytes (data not shown). As for melanoma cells, HLA-A, HLA-B, and HLA-C were well expressed (≥ 50% positive cells) in 12 patients and downregulated (≤ 20% positive cells) in the remaining eight cases. Melan-A/MART-1 was highly expressed (≥ 50% positive cells) in 12 cases, with two tumors showing 40% stained cells and the remaining six cases displaying low expression (< 20% positive cells). Tumors of seven of eight clinical responders (including two with CR, three with SD, and three long-term disease-free individuals) showed high expression of HLA class I (70% to 100% positive cells) and either Melan-A/MART-1 or of gp-100 (HMB-45), whereas among the nonresponders, class I HLA was downregulated (≤ 20% positive cells) or absent in seven of 12 cases (patient 01-006 was not assessable) (Table 4)

Table 4. Immunohistochemical Analysis of the Infiltrate of HLA-A, HLA-B, HLA-C, and Melanoma Antigens in Tumor Tissues Used for Vaccine Production in Assessable Patients, and Clinical and Immunologic Response

Patient No.	Tumor-Infiltrating Lymphocytes				Melanoma Cells†			Clinical Response	T-Cell Response (ELISPOT)
	Hematoxylin and Eosin	CD3*	CD8* (%)	CD45RO* (%)	HLA-A, -B, -C (%)	HMB-45 (%)	MART1 (%)		
01-001	Absent	Rare	Negative	100	0	100	15	PD	NA
01-002	Absent	Rare	60	80	20	100	80	PD	–
01-004	Nonbrisk	Scattered	80	100	Rare	90	40	PD	+
01-006	Absent	Negative	Negative	Negative	NA	100	100	PD	NA
01-007	Brisk	Diffuse	80	100	70	40	50	CR	+
01-009	Nonbrisk	Scattered	100	100	70	80	100	PD	–
01-010	Brisk	Diffuse	70	90	70	80	80	SD	+
01-015	Absent	Rare	100	100	70	100	100	PD	–
01-016	Absent	Rare	100	100	10	100	10	PD	NA
01-017	Absent	Rare	100	100	70	100	100	LTS	+
01-019	Absent	Rare	100	100	70	Rare	70	LTS	+
01-020	Absent	Negative	Negative	Negative	90	10	100	PD	+
01-021	Nonbrisk	Scattered	100	100	Focal	100	Rare	SD	+
01-022	Absent	Rare	Negative	100	Rare	0	0	PD	+
01-023	Absent	Rare	100	100	0	100	80	PD	–
01-025	Absent	Rare	100	100	60	10	Rare	PD	+
01-027	Absent	Negative	Negative	Negative	Rare	Rare	10	PD	–
01-029	Brisk	Diffuse	60	100	80	100	100	PD	–
02-003	Nonbrisk	Focal	100	100	70	10	80	CR	+
03-003	Brisk	Diffuse	70	50	100	15	40	LTS	–
03-005	Brisk	Diffuse	100	100	100	50	5	SD	ND

Abbreviations: LTS, long-term (more than 6 months) DFS; ND, not determined; NA, not assessable because of high background; PD, progressive disease.
*Evaluation of CD3, CD8, and CD45RO refers only to intratumoral lymphocytes. CD8 and CD45RO are given as percentage of $CD3^+$ lymphocytes.
†HLA-A, -B, -C, HMB-45, and MART-1 expression of tumor cells was given as percentage of the entire population of tumor cells.

and Melan-A/MART-1 and/or gp100 in three of 12 cases examined.

DISCUSSION

Our results show that vaccination of metastatic melanoma patients with autologous tumor-derived HSPPC-96 is feasible and well tolerated. In addition, vaccination resulted in some clinical responses apparently associated with T-cell response against melanoma-associated antigens. In the large majority of cases (n = 39), 3 to 4 g of fresh tumor tissue could provide enough HSPPC-96 to vaccinate patients for at least one cycle. Twenty-one of the 39 assessable patients were also given a second cycle of vaccination with four biweekly injections of Oncophage. No treatment-related toxicity (grade I to IV) was found, with the exception of a few instances of local transient erythema caused by injection trauma. Adverse events occurred in six patients, but all were judged to be unrelated to treatment with HSPPC-96.

There were two CRs (patients 01-007 and 02-003), one still in remission 38 months after vaccination and the other recurring after 24 months (as of April 2002). It should be noted, however, that patient 02-003, after 2 months of CR, received low-dose IFN-α therapy, which might have contributed to this result despite the fact that she had already received IFN-α without success before starting vaccination. Three additional patients (01-010, 01-021, and 03-005) showed SD for 191, 272, and 153 days, respectively. Moreover, of the 11 patients made disease-free by surgery at the time of vaccine preparation, three (patients 01-019, 01-017, and 03-003) remained disease-free for 642, 366, and 252 days after vaccination, respectively. Altogether, the median survival time of the 28 tumor-bearing patients was 402 days and median time to progression was 29 days. If one arbitrarily considers the three SDs in addition to the two CRs, 17.8% of the 28 metastatic melanoma patients with measurable disease showed a durable clinical response.

We obtained immunologic evidence (in two patients) that the HSPPC-96 used for vaccination contained melanoma-derived antigenic peptides. This was shown through a re-presentation assay[13] and is the first demonstration of re-presentation of tumor antigens by gp96 in a human system. Because of the lack of autologous melanoma lines, we could not assess whether recognition of autologous HSPPC-96 in these patients involved unique or shared antigens. Such studies are now ongoing. Evidence for association of HSPs with antigenic peptides has been obtained for a large number of antigens[10] including, recently, for HBV-specific epitopes.[29]

In 11 of 23 cases examined, a class I HLA-restricted immunization likely directed to melanoma antigens was shown to occur after vaccination with HSPPC-96, as evaluated by a significant increase or de novo appearance of IFN-γ release by patients' PBMCs exposed to autologous or HLA-A–compatible melanoma cells. A similar frequency of ELISPOT responses was reported by Janetzki et al[16] in 12 patients with different types of nonmelanoma tumors who were immunized with HSPPC-96. In four of four cases tested in our study, clinical response was associated with the T-cell response to melanoma cells (Table 3). The use of peptide/MHC tetramers allowed us to correlate the increased frequency of Melan-A/MART1-specific T cells with clinical CR in patients 01-007 and 02-003 (Rivoltini et al, manuscript in preparation), although, as shown in Fig 3, some patients (01-009, 01-002, 03-006, and 01-029) showed a prior existence of antimelanoma response even before vaccination that might have included T-cell and/or NK-cell activity. Such findings have also been reported in colon cancer and melanoma patients,[30-32] and it can be explained by recognition of tumor antigens occurring, in a sizable number of cases, during the natural history of tumor growth. The lack of apparent T-cell response in approximately 50% of patients (12 of 23) may be attributed to several, nonexclusive factors. These include the lack of antigens in the original tumor, the loss of antigenicity during vaccine preparation, lack of antigen recognition by the host's immune system, or our inability to detect the responses in the assays used. Of these, loss of antigenicity during the vaccine manufacturing appears unlikely, because at least two Oncophage preparations were shown to specifically stimulate autologous PBMCs of vaccinated patients (Fig 3).

Immunohistochemical analysis of the melanomas revealed downregulation of expression of HLA-A, HLA-B, or HLA-C in 46% (nine of 21) of the assessable patients examined, a figure consistent with data reported in the literature for metastatic melanoma.[33] A sample that showed $\leq$ 20% of tumor cells to be HC-10$^+$ was considered to have a downregulated HLA expression. Furthermore, our use of the HC-10, an HLA class I framework mAb, may have underestimated the extent of downregulation of the HLA at the level of single alleles. It is difficult to evaluate the significance of the downregulation of class I HLA, because even in the two patients who showed CRs, a fraction of tumor cells had lost surface expression of HLA. Such class I MHC-negative cells may have been destroyed by bystander killing or may have become targets of the NK response elicited by immunization with gp96, as shown previously.[14,15]

The expression of melanoma antigens was less frequently reduced, with two groups of seven tumors each, out of 21 samples examined, showing less than 20% of cells positive for Melan-A/MART-1 and gp100, respectively. It was of interest that high HLA-A and Melan-A/MART-1 expression was associated with clinical response, suggesting that pa-

tients whose tumors bear the appropriate target molecules (ie, HLA-antigen complexes) may become immunologically primed during early tumor growth and able to develop a recall response on vaccination with HSPPC-96.

This study was initiated as part of a clinical program to translate into human trials some of the extensive data on tumor-protective immunogenicity of tumor-derived HSP-peptide complexes. It has shown that in addition to being feasible, safe, and well-tolerated, immunization of cancer patients with bulky advanced disease with HSPPC-96 is associated with clinical and measurable immunologic responses in a significant minority of patients. These data, although limited in number and scope, are consistent with the murine experience that therapy of mice with progressively growing cancers with gp96-peptide complexes leads to regression of tumors in a small number of mice and stabilization of disease in additional mice.[14]

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European Journal of Immunology Highlights

MINI-REVIEW:

Message in a bottle: Role of the 70-kDa heat shock protein family in anti-tumor immunity

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Extracellular heat shock protein 70 (HSP70) is a potent agent for tumor immunotherapy, which can break tolerance to tumor-associated antigens and cause specific tumor cell killing by cytotoxic $CD8^+$ T cells. The pro-immune effects of extracellular HSP70 are, to some extent, extensions of its molecular properties as an intracellular stress protein. The HSP70 are characterized by massive inducibility after stress, preventing cell death by inhibiting aggregation of cell proteins and directly antagonizing multiple cell death pathways. HSP70 family members possess a domain in the C terminus that chaperones unfolded proteins and peptides, and a N-terminal ATPase domain that controls the opening and closing of the peptide binding domain. These properties not only enable intracellular HSP70 to inhibit tumor apoptosis, but also promote formation of stable complexes with cytoplasmic tumor antigens that can then escape intact from dying cells to interact with antigen-processing cells (APC) and stimulate anti-tumor immunity. HSP70 may be released from tumors undergoing therapy at high local extracellular concentrations, and send a danger signal to the host leading to APC activation. Extracellular HSP70 bind to high-affinity receptors on APC, leading to activation of maturation and re-presentation of the peptide antigen cargo of HSP70 by the APC. The ability of HSP70-peptide complexes (HSP70-PC) to break tolerance and cause tumor regression employs these dual properties as signaling ligand and antigen transporter. HSP70-PC thus coordinately activate innate immune responses and deliver antigens for re-presentation by MHC class I and II molecules on the APC cell surface, leading to specific anti-tumor immunity.

Key words: Heat shock protein 70 · Tumor immunity · APC · Internalization · Immunogenic peptides

Introduction: extracellular HSP70 and its role in anti-tumor immunity

HSP70 and other molecular chaperones are finding increasing and effective use in tumor immunotherapy [1–4]. One of the remarkable aspects of these approaches is the variety of contexts in which extracellular HSP are deployed in tumor immunotherapy. HSP70 in some approaches has been employed as an adjuvant in combination with other treatments to activate APC, and break tolerance to tumor-associated antigens, while in others it is used primarily as a carrier protein to deliver extracellular antigens to MHC class I and II molecules on APC [1, 5–7]. The antigens presented by HSP70 can be individualized to patients' tumors, or may be common to many tumor types [2, 8]. The variety of ways in which the HSP70 may be deployed appears to reflect the molecular properties of HSP70,

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Abbreviations: HSC: heat shock cognate · **PC:** peptide complexes

and the multiple ways in which it can interact with APC. Here, we discuss the unique properties of the HSP70 family that make them effective agents in capturing and cross-presenting tumor antigens.

The HSP70 family is intrinsic to cellular life, permitting proteins to perform essential enzymic, signaling and structural functions within the tightly crowded milieu of the cell, and working to avert the catastrophe of protein aggregation during stress [9, 10]. HSP70 is induced to extremely high levels by stress along with a cohort of other HSP through powerful transcriptional activation, mRNA stabilization and preferential translation [9]. For molecular chaperone function, HSP70 family members are equipped with two major functional domains, including a C-terminal region that binds peptides and denatured proteins, and an N-terminal ATPase domain that controls the opening and closing of the peptide binding domain (for review see [11]). These two domains play important roles in the function of HSP70 in tumor immunity, mediating the acquisition of cellular antigens and their delivery to immune effector cells [7, 12]. There are at least 12 members of the human HSP70 family, including proteins expressed in the cytoplasm, endoplasmic reticulum (ER) and mitochondria [9, 11, 13]. Both constitutive [such as human heat shock cognate (HSC) 73] and stress-inducible HSP family members (human HSP72) exist [13, 14]. HSP70 expression becomes dysregulated in many types of cancer, leading to elevated HSP70 levels under non-stress conditions that protect emerging cancer cells from the apoptosis that accompanies many steps in transformation, but also creates an opportunity for vaccine design [13, 15–17]. A pro-immune function for HSP70 family members may also be inferred from the fact that the immunosuppressive drug 15-deoxyspergualin binds with high affinity to HSP70 proteins [18].

Role of HSP70 family members in antigen processing and capture

In most mammalian cells, damaged, denatured or superfluous proteins are degraded through the ubiquitin proteasome pathway to small peptides, subsequent to release and complete breakdown to amino acids (Fig. 1) [19, 20]. A fraction of the peptides released from the proteasome are, however, not degraded further and are instead used for immune surveillance purposes [19–21]. Such peptides are taken up into the ER through an ABC family transport system that involves the transporters associated with antigen processing 1 (TAP1) and TAP2 proteins (Fig. 1). TAP1 and TAP2 form a complex that transports peptides across the ER membrane and delivers them to MHC class I protein complexes [22, 23]. Peptides of suitable size and sequence are then bound by MHC class I, transported to the cytoplasm via the vesicular system and displayed on the cell surface where they are subject to surveillance by cytotoxic, $CD8^+$ lymphocytes [24]. The purpose of such cell surface display of antigens via the MHC class I pathways permits identification of non-self foreign antigens in microorganism-infected cells, which are then targeted for lysis by CTL. However, this pathway is a potential target in cancer therapy if tumor antigens can be targeted for recognition by CTL [12, 25–27]. HSP70 family members have the potential to participate in this pathway for antigen processing and presentation due to their ability to bind peptides using the C-terminal polypeptide binding domain [11, 28–30]. It has been assumed that HSP70 proteins might bind peptides released into the cytoplasm from the proteasome in a similar way to their acquisition by the TAP1/TAP2 complex. This is largely inferred from the fact that HSP70 extracted from tumors can be used to cross-present (see Fig. 1) tumor antigens on APC, which are recognized by specific clones of cytotoxic $CD8^+$ lymphocytes [6, 12, 31]. The peptides that bind to the TAP complex and MHC class I, although of varying

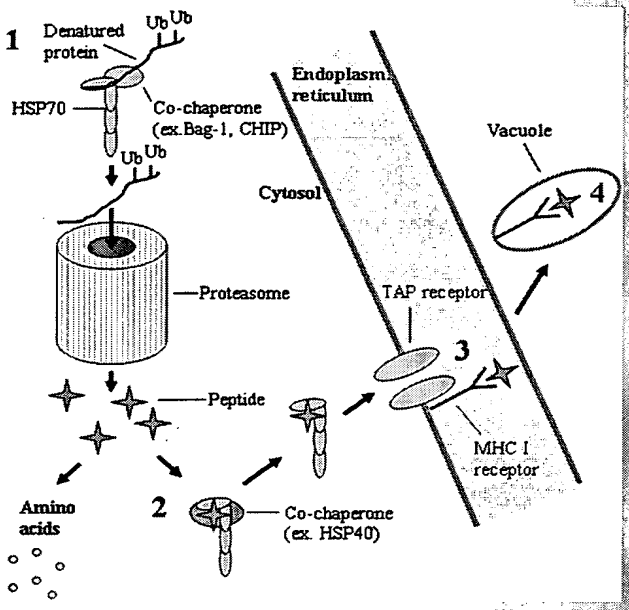

Fig. 1. Potential role of HSP70 in antigen processing and presentation on MHC class I molecules. HSP70 appears to play at least two roles in antigen processing including: (1) delivery of ubiquitinated proteins to the proteasome, and (2) chaperoning of peptides exiting the proteasome. Chaperoned peptides may thus be protected from endopeptidases, and (3) passed onto TAP1/TAP2. The TAP complex can then load peptides onto the MHC class I complex, and (4) the MHC class I-PC can then traverse the ER and be inserted into the plasma membrane.

sequence, have some common properties; such peptides are 8–10 amino acids in length [32, 33]. Most MHC class I ligands bind in extended conformation to the MHC binding groove and have an anchor residue at the C terminus that is either hydrophobic or basic, and such sequences are also preferred by TAP [26, 33]. MHC class I-peptide binding is of fairly low affinity (K_D 10^{-6} M), but is almost irreversible in the intact MHC class I-peptide complex. The binding domain of HSP70 has common properties including a similar affinity for peptides (K_D approx 10^{-6} M; L. Mannheim-Rodman and S. K. Calderwood, unpublished) and accommodates peptides in extended conformation of 7–15 amino acids [34–36]. A number of studies have addressed the peptide sequence binding preferences of HSP70 proteins and indicated a similar peptide binding preference for MHC class I, indicating roles for hydrophobic and basic amino acids [30, 34–36]. Inferences derived from these studies, however, are complicated by the findings that substrate preferences in HSP70 family proteins are determined not solely by the properties of the peptide binding domain of HSP70 itself, but by J-domain proteins, co-factors that localize HSP70 to target molecules and effect binding by modulating the ATPase activity of HSP70 (Fig. 1) [37, 38]. A range of J domain proteins with different substrate preferences have been found [11, 39–41]. Thus, peptide binding by HSP70 proteins may be determined at least partially by the J domain protein partner [38]. There is an interesting confluence in properties between MHC class I, MHC class II and HSP70 in that each protein requires the assistance of a co-factor for peptide association; TAP1 is required for MHC class I-peptide binding, HLA-DM for MHC class II and J domain proteins for HSP70. It is currently unknown whether HSP70 binds preferentially to any class of intracellular peptides. However, in an *in vivo* proteomic study, Grossmann et al. [42] suggest that HSP70 binds to peptides of generally 8–26 amino acids in length. Furthermore, HSP70 favorably interacts to a 5-amino acid core sequence, which likely contains some acidic residues [42]. One compelling hypothesis is that a fraction of the intracellular HSP70 binds peptides released into the cytoplasm from the proteasome, protects them from further degradation and passes them to the TAP1/TAP2 complex. It is known that HSP70 proteins function "upstream" in the processing of proteins. HSP70 can bind to damaged target proteins, while associated with CHIP (C terminus of HSC70-interacting protein), which functions as an HSC70 binding ubiquitin E3 ligase, marking the target protein for proteasomal degradation by ligation of its side chains with ubiquitin [43–45]. This complex is then transported to the proteasome in the company of another HSP70 binding protein Bag-I [44]. HSP70 may then perform an "end-around" and accept and chaperone peptides extruded from the exit tunnel of the proteasome.

In addition, HSP70 family member HSC73 takes part in another form of antigen processing; HSC73 is evidently required for the processing of external antigens in the lysosome proximal endosome compartments, and is thus involved in the processing of peptides that associate with MHC class II proteins in APC and activate immune response through CD4 T lymphocytes [46]. HSC73 co-associates with MHC class II in spherical organelles in macrophages and leads to the presentation of external antigens to MHC class II-restricted T lymphocytes [46]. A role for HSC73 in protecting peptides generated by proteolysis in MHC class II-containing endosomes from further breakdown has been suggested, which is similar to the role proposed for the HSP70 family in protecting cytoplasmic peptides [47–49]. The presence of HSC73 in clathrin-coated pits present in endosomes and lysosomes is well established and HSC73 has been shown by Dice and co-workers [47–49] to participate in targeting proteins for degradation through recognition of a consensus sequence (KFERQ). A common theme for HSP70, of mobilizing target proteins towards the sites of degradation while sparing a fraction of the partially digested peptide for immune surveillance, is suggested for both main pathways of protein degradation in the cytoplasm (proteasome) and lysosome. As the lysosomal pathway can be stimulated by serum starvation of tissue culture cells, it may thus play a role in generation of tumor immunity in nutritionally deprived tumor cells, which could potentially be captured in the production of anti-tumor HSP70 based vaccines [48]. A major unknown in these studies is, whether, HSP70 proteins capture a nonspecific sample of intracellular peptide or actively select classes of peptides along structural lines, guided perhaps by associated J-domain proteins. In this context it has been shown recently that immunosuppressive drug 15-deoxyspergualin (DSG) specifically binds with high affinity to HSC70 and inhibits interactions that do not require DnaJ co-chaperone activity [18, 50]. Although these studies are not definitive, as the properties of DSG are not completely understood, they do indicate a need to understand the mechanisms of molecular chaperone-peptide interactions in antigen processing.

Escape of HSP70 proteins from the cytoplasm; insertion onto the membrane and release into the external milieu

The findings that HSP70 is released into the blood stream and stimulates the production of anti-HSP70 antibodies after a number of pathological conditions

was the first indication that extracellular HSP70 may play some physiological role [51–53]. It has been suggested that circulating HSP70 is (a) derived from cells dying under a number of pathological conditions or (b) actively released from intact cells [53, 54]. Approaches to cancer therapy aim to destroy tumors locally by a gradual necrotic mechanism with the aim of producing HSP70 release at high concentrations [1, 4, 55, 56]. The rationale behind this approach rests on the high constitutive levels of tumor HSP70 and abundant induction of *HSP70* gene transcription (an unusually efficient process) by the stress of therapy [13]. The terminal stages of necrosis may also favor antigen uptake by HSP70, as intracellular ATP levels decline and peptides become locked onto the ADP-associated HSP70 [57]. The extracellular presence of HSP70 can be enhanced by heat shock in prostate cancer cells [58]. HSP70-peptide complexes (HSP70-PC) released locally in the tumor microenvironment may thus constitute a "danger signal" and attract and activate APC [59, 60]. Such HSP70 is likely released along with other molecular chaperones with pro-immune activity and other danger signals, such as the high-mobility group-1 protein (HMG-b1) ([59] and S. Mambula and S. K. Calderwood, in preparation). HSP-PC are, however, not merely danger signals but likely carry samples of tumor-associated antigens, which may be fragments of mutated proteins or re-expressed embryonic or developmental antigens for potential cross-presentation to immune effector cells [61]. Injection of HSP70 or multi-chaperone based vaccines may mimic these effects [2, 62]. These mechanisms are, to some extent an over-simplification, as tumor cell populations under therapy rarely die from a single form of cell death and necrosis, apoptosis and other pathways likely occur in concert. However, it has also been shown that HSP70 over-expression in the presence of slow necrotic death is an extremely potent approach to breaking tolerance and induction of specific immune destruction of tumors [1].

As mentioned above, little is known about how HSP70, a protein with no signal sequence for secretion, exits cells by mechanisms other than escape from cells undergoing necrotic lysis. However, Dice *et al.* [63, 64] showed that proteins can be released from a late endosomal lysosomal location where HSC70 participates in protein degradation (HSC70 is the murine homologue of human HSC73). In addition, HSC70 can associate with the transferrin receptor in exosomes, suggesting a potential exosomal mechanism for HSC70 release [65]. Secretion and/or detergent-resistant microdomain fractions with associated HSP70 have also been observed in epithelial cells in normal conditions, and a lipid raft-based mechanism has been suggested for membrane delivery and release of HSP70. [66]. Further studies are currently needed to define the mechanisms of HSP70 release from cells under stress or non-toxic conditions.

In addition to release into the tumor microenvironment, HSP70 also becomes associated with the extracellular face of the plasma membrane [67–70]. Cell surface HSP70-PC in tumor cells could interact with receptors on APC in this context, or be released into the microenvironment. A number of studies show that HSP70-PC associated with the cell surface of tumors interacts with T cell receptors on $CD3^+$, $CD4^-$, $CD8^-$ natural killer (NK) cells and suggest that cell surface HSP70 can present processed antigen to such receptors [70]. It may thus be significant that a granzyme B mediates NK cell killing by mechanisms involving membrane-bound HSP70-PC of tumor cells, thus supporting a significant role of HSP70 in NK cell function [69]. Recent attempts to characterize the global profile of the cell surface proteome of cancer cells also reveal the abundant presence of at least two HSP70 members on the cancer cell surface [71].

Binding of HSP70-PC to the cell surface of APC

HSP-PC released from tumor cells undergoing necrosis will be of necessity diluted as they enter the interstitial fluid, and APC response to such low concentrations of HSP70-PC implies the existence of high-affinity receptors. Extracellular HSP70-PC have at least two major enhancing effects on APC function: (i) induction of innate immune function through activation of the APC maturation program [12, 72], and (ii) induction of the adaptive immune response, leading to the transport of peptide antigens into APC cells and delivery to MHC molecules [6]. Whether these two effects are achieved through binding to one or more cell surface receptors is not clear. A major emphasis has therefore been placed on identifying HSP70 receptors on the APC surface. Previous studies indicated HSP-PC-mediated uptake of tumor antigens through receptor-mediated endocytosis because of the low (nanomolar) concentrations of HSP-PC involved and the saturability of the process [73–75]. Although the nature of the high affinity receptors for HSP70-PC binding to the APC cell surface has still not been fully defined, four main classes of cell surface structure have been suggested, including: (i) the CD14/TLR 2/4 complex, (ii) the CD91 receptor, (iii) CD40, and (iv) scavenger receptors, most notably the lectin-like receptor for oxidized low-density lipoprotein (also known as LOX-1) [6, 57, 73, 76]. The CD14/TLR 2 or 4 pathway, which is known to mediate cellular responses to bacterial danger signals, such as LPS and peptidoglycans, appears to play a role in activation of the innate immune response gene (TNF-α, IL-1β, IL-6) expression

program by HSP70, [12, 73, 77, 78]. However, there is currently only evidence for a functional interaction between HSP70, CD14, TLR2 and TLR4. Others have suggested a role for binding of HSP70 and other immune-effective stress proteins to APC cells through CD91, the α2-macroglobulin receptor, and have proposed this structure as the sole receptor for HSP70-PC uptake and presentation to MHC molecules in immune effector cells [79]. In addition, a recent study by Becker *et al.* [57] also shows evidence for binding of HSP70-PC to another receptor, the TNF receptor family protein CD40. This interaction is stabilized by the presence of ADP and peptide bound to HSP70 [57]. However, other studies indicate instead that only mycobacterial HSP70 is able to bind CD40 and that mammalian HSP70-PC does not directly interact with CD40, casting doubt on a role for CD40 as a direct receptor for mammalian HSP70 [76, 80]. Finally, Delneste *et al.* [57] have shown a significant role for the c-type lectin LOX-1 in HSP70 binding and antigen cross-presentation, and this receptor seems a convincing candidate for mediation of immune effects of HSP70. We have directly compared HSP70 binding to each class of receptor after overexpressing them in non-APC cells [81]. Our studies indicate that only the scavenger receptors, as exemplified by LOX-1, bind with high avidity to mammalian HSP70, while TLR2, TLR4, CD40 or CD91 expression failed to support binding [81].

Clearly, despite these promising findings, much has to be learned regarding the nature of the proximal HSP70 receptor or receptors responsible for the immune effects of HSP70. One perplexing question is how cells of the immune system recognize the wide range of stress proteins from a number of unrelated families evidently capable of activating APC. One possibility is that the peptide component of the HSP70-PC complex plays an important role in recognition by HSP70 receptors, in a similar way to recognition of MHC-PC by T cell receptors. In this context, the finding that cell surface expressed HSC70 can interact with the rat Vδ6 T cell receptor on NK cells and present peptides to these receptor cells may be of significance for HSP70-APC interactions [70, 82]. Another possibility is that the nature of the adenosine phosphate moiety bound to HSP70 (ATP *vs.* ADP) may be important in the interaction with a receptor. However, scavenger receptors can bind a wide array of unrelated ligands by unknown mechanisms (and have been described as "molecular flypaper") making the promiscuous association with molecular chaperones from different families perhaps more understandable [83]. As will be gathered, understanding of HSP70 recognition by cell surface structures in APC is still not completely understood and may await a more complete determination of authentic HSP70 receptors.

Internalization of HSP70-PC by APC, activation of transmembrane signaling cascades and presentation of peptides to cell surface MHC molecules

HSP70-induced APC maturation and pro-inflammatory signaling

Binding of HSP70-PC is succeeded by the activation of transmembrane signaling mechanisms, internalization and the delivery of the peptide cargo of HSP70 to MHC molecules. Whether a single receptor carries out these processes or whether multiple receptors mediate these effects is not clear.

HSP70 can initiate a potent innate immune response resulting in APC maturation as well as a pro-inflammatory response (Fig. 2) [1, 2, 84]. Dendritic cell (DC) maturation requires the activation of a gene expression program that leads to production of co-stimulatory molecules including CD40, OX40L, B7.1 and B7.2 on the cell surface for effective interaction with $CD8^+$ T lymphocytes (Fig. 2) [85]. Direct HSP70 binding to the CD40 receptor would be an attractive hypothesis as ligation of CD40 plays an important role in DC maturation [85]. In fact, a key functional role for CD40 in breaking tolerance in an autoimmune form of diabetes by HSP70 has been demonstrated [72]. However, as mentioned earlier, it is not clear whether direct HSP70-CD40 binding to the APC cell surface is or is not involved. It seems likely that that HSP70-induced DC

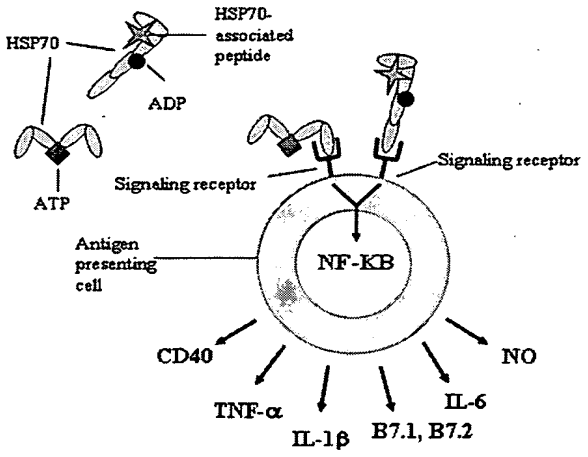

Fig. 2. Mechanism of HSP70-induced innate immune response (pro-inflammatory and co-stimulatory molecule expression). ATP- or ADP-bound HSP70 binds to signaling receptor(s) present on APC. All signaling pathways converge to the activation of the NF-κB pathway. The result of this activation is the stimulation of cytokine (TNF-α, IL-1β and IL-6), co-stimulatory molecule (B7.1, B7.2, CD40 and MHC class II) and nitric oxide (NO) release.

maturation involves CD40 and/or CD40L up-regulation, and probably requires activation of the transcription factor NF-κB [86]. Most of the genes involved in DC maturation require the activation of NF-κB and, therefore, receptors that are involved in DC maturation by HSP70 likely cause downstream activation of NF-κB (Fig. 2) [86]. Some intriguing studies have shown that extracellular HSP70 induces NF-κB through the activation of the CD14/TLR signaling pathway in a CD14-dependent manner in APC [73, 87]. This results in the expression of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 as well as co-stimulatory molecule B7.1 [73, 87]. Although the role of this pathway in the innate immune response to HSP70 is not clear, it can be activated equally effectively by HSP70-PC and free HSP70 [88]. However, this effect seems indirect since there is no evidence for high-affinity binding of extracellular HSP70 to TLR 2, TLR 4 or CD14 [81]. In addition, some skepticism has been directed at the role of CD14 or TLR due to potential HSP70 contamination by endotoxin in these effects [89]. In addition, other potential HSP70 receptors are coupled to NF-κB, and LOX-1 has recently been shown to activate the NF-κB pathway in endothelial cells stimulated by oxidized-LDL, while ligation of CD40 can lead to NF-κB activation in APC [90, 91]. Oxidized-LDL can also induce CD40 expression through LOX-1 in the same cells suggesting a potential role for LOX-1 in APC maturation by HSP70 [92].

HSP70-PC internalization by APC and peptide association with cell surface MHC molecules

A receptor-mediated mechanism appears to be involved in antigen cross-presentation by HSP [7, 93]. After binding HSP70-PC, such a receptor would mediate internalization of complexes and delivery of the peptide cargo to MHC class I and perhaps MHC class II molecules. Receptor-mediated protein internalization is mainly governed through specific motifs located in the cytoplasmic tails of receptors which engage the protein coats of receptor-rich regions of membranes that ultimately give rise to the coated vesicles that mediate internalization [94, 95]. Tyrosine-based or di-leucine-based sorting signals within the C termini of receptors represent the sorting codes for the vast majority of receptor endocytosis and target liganded receptors to specific organelles [94, 95]. Tyrosine-based sorting signals usually involve short consensus internalization motifs such as YXXΘ (Θ represents an amino acid with a bulky hydrophobic side chain, X is any amino acid) and NPXY [94, 95]. Each motif is recognized by components of protein coats and is targeted to specific compartments such as endosomes [94, 95]. YXXΘ and di-leucine motifs are recognized by the adaptor protein complexes (AP-1,2) and NPXY motif through clathrin, AP-2 and Dab2 [94, 95]. In some circumstances, phosphorylation of the single tyrosine present on these motifs is observed, and can regulate the signal recognition to endocytic component as well as the initiation of specific signal transduction pathways [94, 95]. Among the proposed HSP70 receptors, only CD40 has been shown directly to take part in HSP70-PC uptake [57]. Nevertheless, CD91 and LOX-1 also mediate internalization of various extracellular ligands [96–98]. Since both receptors are involved in HSP70-mediated antigen cross-presentation, CD91 and LOX-1 may also possess the capacity to facilitate internalization of HSP70-PC. Interestingly, the uptake of one CD91 ligand, receptor-associated protein (RAP), is mediated mainly through a YXXL motif [98]. While it has been shown that LOX-1 can internalize oxidized LDL, the mechanisms that underlie LOX-1 triggering of oxidized LDL uptake are not clear [99]. However, some information regarding LOX-1-mediated internalization may be partially deduced from ligand uptake studies of the closely related c-type lectin receptor Dectin-1. Dectin-1 utilizes the YXXL motif to endocytose yeast zymosan [100] and thus, by implication, YXXL motif could play a role in HSP70-PC uptake.

Following receptor-induced extracellular HSP70-PC internalization by APC, complexes may traffic through a number of intracellular compartments, leading to peptide release into the cytoplasm and re-presentation on the cell surface associated with MHC proteins (Fig. 3). HSP70-PC may be taken up by the endocytic

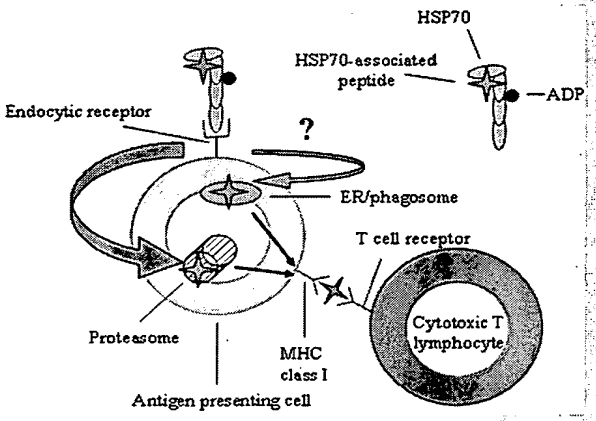

Fig. 3. Proposed model for HSP70-mediated antigen cross-presentation (adaptive response). A repertoire of HSP70-associated antigens (HSP70-PC) elicits specific adaptive immune responses. HSP70-PC bound to endocytic receptors is internalized by APC through the activation of an intracellular signaling module. In APC endocytic compartment, HSP70-PC is transported to the proteasome or the ER/phagosome compartment where the peptide is processed and transferred onto MHC class I molecule for representation on the cell surface. The MHC class I/antigen complex is recognized by cytotoxic T lymphocyte bearing an antigen-specific T cell receptor.

pathway and lead to peptide presentation to MHC class II molecules by the standard extracellular pathway of antigen presentation [101]. In addition, HSP70 can also deliver peptides to MHC class I molecules through the re-presentation or cross-presentation pathways [102]. After internalization of HSP70-PC, peptides may be released into the cytoplasm and processed, as previously mentioned in the Introduction to MHC class I molecules by the classical antigen re-presentation pathways. However, cross-presentation may proceed through a newly discovered alternative route involving a specialized MHC class I structure known as the ER/phagosome fusion compartment, which seems auto-sufficient to induce antigen cross-presentation (Fig. 3; discussed in [103]). HSP70 and HSP90 have been located in this compartment, supporting a potential role for HSP in this pathway of antigen cross-presentation [103]. The possibility that HSP70 receptor(s) can also co-localize with components located in this structure during HSP70-mediated cross-presentation is thus worth exploring. Intriguingly, cross-presentation of cell-associated antigens to MHC class I is significantly impaired in cells devoid of the *hsf1* gene [104]. In this context, reduction of intracellular HSP70 and HSP90 content by *hsf1* inactivation correlates with loss of antigen-mediated cross-presentation, re-enforcing a key role for HSP70 and/or HSP90 in this process [104]. So far, no definitive studies have addressed the pathways of HSP70-PC internalization involved in tumor antigen cross-presentation. Nonetheless, the internalization pathway of another HSP family member, gp96, has been studied in the past few years [93, 105]. Gp96-PC is rapidly internalized, after interacting with undefined cell surface receptor(s), into a pre-endosomal compartment [93, 105]. Such endocytosed Gp96 was found to co-localize with FcR and MHC class I but not with other receptors such as CD91, transferrin, rab5a or lysosomal markers such as LAMP-1 or LAMP-2 [74, 105].

Conclusion

Extracellular HSP70-PC is an effective agent for breaking tolerance to tumor antigens and eliciting specific $CD8^+$ tumor-specific immunity. Targeting HSP70 is thus a highly effective approach to tumor immunotherapy. The pro-immune effects of extracellular HSP70 appear to reflect its ability to act as both a signaling ligand to induce APC maturation and inflammatory processes, and as a carrier protein to chaperone and transport tumor antigens for re-presentation by APC. However, most of the molecular mechanisms that underlie these effects are not completely understood and further progress in the field will likely require a clearer understanding of HSP70-PC release from tumor cells and interaction with immune effector cells. Areas that require development include: the role of HSP70 and co-chaperones in antigen processing in tumor cells, mechanisms of release of HSP70-PC from tumors, receptor-mediated uptake of HSP70-PC by APC, and mechanisms of HSP70-mediated re-presentation of tumor antigens to immune effector cells. Understanding these processes may permit us to manipulate more effectively the use of HSP70 in tumor immunotherapy.

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